CERTIFICATE OF ELECTRONIC SUBMISSION

January 11, 2007

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Michael G. Rosenblum	Group Art Unit: 1642
Serial No.: 10/676,725	Examiner: Goddard, Laura B.
Filed: October 1, 2003	Atty. Dkt. No.: CLFR:029USD1
For: NOVEL ANTIBODY DELIVERY SYSTEM FOR BIOLOGICAL RESPONSE MODIFIERS	

SUPPLEMENTAL APPEAL BRIEF

MS Appeal Briefs
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Appellants hereby submit this Supplemental Appeal Brief to the Board of Patent Appeals and Interferences pursuant to the Notice of Non-Compliant Brief dated December 27, 2006 as well as 37 C.F.R. §41.31(a)(1) and 41.37 in light of the Final Office Action dated July 18, 2006. It is believed that the filing of the present Supplemental Appeal Brief is timely. However, if any fees are due for any reason relating to the enclosed materials, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/CLFR:029USD1.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, Research Development Foundation. The subject matter of the application is currently licensed to Targa Therapeutics Corporation.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-6, 8-9, 11-12, 20 and 22 are cancelled. Claims 15 and 17-19 are withdrawn. Claims 7, 10, 13-14, 16, 21, and 23 – 32 are currently under examination, are subject to rejection and are the subject matter of the present appeal.

IV. STATUS OF AMENDMENTS

An amendment is filed concurrently herewith to correct the dependencies of claims 23 and 24, each of which should depend from base claim 26. This should readily address the section 112, second paragraph concerns.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

Claim 26 is the main claim, and is directed to a method of treating cancer in a human patient in need of such treatment, wherein the method comprises steps of (a) identifying a patient having a tumor, which tumor comprises cells for targeting and wherein those cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites; (b) obtaining a composition comprising a protein with an antigen recognition site directed toward a cell surface associated antigen conjugated or fused to the biological response modifier, wherein it has been determined that cells of the patient's cancer express an antigen recognized and bound

by the protein with an antigen recognition site; and (c) administering an amount of the composition to the patient effective to treat the cancer. The foregoing is clearly described in the application Summary, page 4, line 10 (third full paragraph), through page 7, line 9 (second full paragraph), particularly page 4, line 22, to page 5, line 6 (the paragraph bridging pages 4 and 5) and the page 5, lines 7-12 (first full paragraph, page 5); see also Detailed Description, page 9, line 2, to page 16, line 23 (first paragraph, to page 16, last paragraph), particularly page 15, lines 3-8 (the first full paragraph, page 15). These later passages specifically disclose the concept of targeting an antigen contained on a patient's tumor, wherein the "cell surface antigenic marker [is expressed] at concentrations in excess of that found at other non-target sites."

Claims 7 and 29-31 concern targeting cancers selected from the group consisting of breast cancer, cervical carcinoma and melanoma. See specification, page 5, line 13, to page 6, line 3 (paragraph bridging pages 5 and 6).

Claims 10, 21 and 25 concern cancers that express an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009), and further wherein the protein is a monoclonal antibody that recognizes and binds the antigen. See specification, Example 4, page 19, line 1, through page 20, line 26.

Claim 13 concerns wherein the biological response modifier is a cytokine, particularly TNF (claim 14), and more particularly TNF-beta (claim 15) or TNF-alpha (claim 16). See specification, page 9, lines 1-13.

Claim 23 concerns fusion of the antigen binding site to the biological response modifier, whereas claim 24 concerns conjugation to the biological response modifier. The difference is that "fusion" typically involves fusions of the respective genes such that expression gives rise to

a single continuous peptide, whereas conjugation typically (but not always) refers to chemical conjugation. See specification, page 9, lines 1-13.

Claim 27 concerns wherein the patient is diagnosed as having a tumor with a specific antigenic determinant that will allow targeting and concentration of the biological response modifier at the site where it is needed to kill tumor cells. See specification, page 15, lines 3-8.

Claim 28 concerns wherein the protein is an antibody (specification, page 6, lines 14-19), further defined as a monoclonal antibody (claim 29; page 4, lines 10-21).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are four rejections that are the subject of the present appeal:

1) A rejection of claims 7, 10, 13, 14, 16, 21 and 23-32 as lacking written description under 35 U.S.C. §112, first paragraph;

2) A rejection of claims 7, 10, 13, 14, 16, 21, and 23-32 as lacking enablement under 35 U.S.C. §112, first paragraph;

3) A rejection of claims 7, 26-29 and 32 as unpatentable under 35 U.S.C. §103 over Oldham *et al.* ("Oldham"; Exhibit 1) in view of Freeman *et al.* ("Freeman"; Exhibit 2);

4) A rejection of claims 13, 14 and 16 as unpatentable under 35 U.S.C. §103 over Oldham *et al.* in view of Freeman *et al.*, further in view of Ferris *et al.* ("Ferris"; Exhibit 3) and Bregman *et al.* ("Bregman"; Exhibit 4); and

5) A rejection of claim 23 as unpatentable under 35 U.S.C. §103 over Oldham *et al.* and Freeman *et al.* in view of Anderson *et al.* ("Anderson"; Exhibit 5).

VII. ARGUMENT

A. Section 112, First Paragraph, Rejections

1) Written Description

In the final Action each of claims 7, 10, 13, 14, 16, 21 and 23-32 were rejected separately under the written description and enablement sections of section 112. We will first address the written description rejection.

To the extent that the written description rejection is understood, it appears as though the Examiner's concern is that in terms of the antibody portion of the immunotoxin, the claims are generic in scope and would cover any antibody capable of recognizing a cell surface epitope that is expressed higher on tumor cells than on normal cells. The Examiner concludes that the specification only discloses two such antibodies (ZME-018 and MoAb 15A8), and that to cover other antibodies, the specification must set forth identifying characteristics (amino acid sequence, etc.) for all such antibodies to be covered. We traverse the foregoing rejection.

We would first note that the Examiner has failed to provide any evidence whatsoever to support a conclusion that one of skill would not be apprised that the present inventors had possession of the full scope of the claimed invention as of the earliest filing date. Thus, in the absence of such evidence, clearly no *prima facie* case has been made.

First, the specification is replete with disclosure which demonstrates convincingly that the present inventors fully appreciated and contemplated the generic scope of the present invention. Evidence of this is discussed above in Section V. Furthermore, the use of immunotoxins to deliver therapeutic agents and/or toxins to targeted tumor cells is generally well-known in the art as of the present filing date, as evidenced by the present specification:

> [0009] Antibodies are proteins normally produced by the immune system of an animal in response to foreign antigens or antigenic determinants. Antibodies bind to the specific antigen to which they are directed. Monoclonal antibodies directed to specific antigens or antigenic determinants may be prepared in large quantities. Monoclonal antibodies to tumor associated antigens localize in tumors after systemic administration to patients with cancer.
>
> [0010] Antibodies, coupled to drugs, have been used as a delivery system by which the drug is targeted to a specific tumor cell type against which the antibody is directed. The linking of cytotoxic agents to antibodies to make "immunotoxins" has been reported. Of particular interest have been immunotoxins of monoclonal antibodies conjugated to the enzymatically active portions (A chains) of toxins of bacterial or plant origin such as Ricin or Abrin. Nevelle and York, Immunol. Rev. (1982) 62: 75-91; Ross et al., European J. Biochem. (1980) 104; Vitteta et al., Immunol. Rev. (1982) 62: 158-183; Ross et al., Cancer Res. (1982) 42: 457-464; Trowbridge and Domingo Nature (Cond.) (1981) 294: 171-173. Immunotoxins have been prepared by conjugating MoAbs with toxins or fragments of toxins derived from plants. Gelonin and ricin are among the most active plant derived toxins in inhibiting protein synthesis.
>
> [0011] Although antibodies have been used as delivery systems for toxic moieties of plant toxins and other cytotoxic drugs, conjugation of antibodies to biological response modifiers such as tumor necrosis factor and the use of such conjugates as specific delivery system to target tissues or cells has not heretofore been possible.

Specification, paragraphs [0009] – [0011].

The Examiner is incorrect legally as well, relying on cases such as *Enzo Biochem, Inc. v. Gen-Probe Inc.*, 296 F.3d 1316 (Fed. Cir. 2002), *University of California v. Eli Lilly and Co.*, 119 F.3d 1559 (Fed. Cir. 1997) and *Noelle v. Lederman*, 355 F.3d 1343 (Fed. Cir. 2004). Each of these cases stand for the well-accepted proposition that where a novel composition is the subject of the claims at issue, the written description requirement obligates the specification must fully and adequately identify and distinguish the novel subject matter. In the *Noelle* case the court observed that the claim at issue was directed to a novel antibody and covered both human and mouse forms of the antibody, and held that the human antibody claims were not adequately

described where the specification only described the mouse version of the underlying antigen. *Id.* at 1349-50.

However, the Federal Circuit has consistently distinguished the foregoing situation, involving the description requirements for novel biological composition, from the present situation where the claim elements being challenged are well-known in the art. Instructive in this regard is the Federal Circuit's recent decision in *Capon v. Eshhar v. Dudas*, 418 F.3d 1349, 76 USPQ2d 1078 (Fed. Cir. 2005), distinguishing *Enzo Biochem* and *Eli Lilly*. As the *Capon* court points out, there is no requirement under written description that a specification contain a detailed description of elements where those elements are well known to those in the field:

> The Board stated that "controlling precedent" required inclusion in the specification of the complete nucleotide sequence of "at least one" chimeric gene. Bd. op. at 4. The Board also objected that the claims were broader than the specific examples. Eshhar and Capon each responds by pointing to the scientific completeness and depth of their descriptive texts, as well as to their illustrative examples. The Board did not relate any of the claims, broad or narrow, to the examples, but invalidated all of the claims without analysis of their scope and the relation of claim scope to the details of the specifications.
>
> Eshhar and Capon both argue that they have set forth an invention whose scope is fully and fairly described, for the nucleotide sequences of the DNA in chimeric combination is readily understood to contain the nucleotide sequences of the DNA components. Eshhar points to the general and specific description in his specification of known immune-related DNA segments, including the examples of their linking. Capon points similarly to his description of selecting DNA segments that are known to express immune-related proteins, and stresses the existing knowledge of these segments and their nucleotide sequences, as well as the known procedures for selecting and combining DNA segments, as cited in the specification.
>
> Both parties argue that the Board misconstrued precedent, and that precedent does not establish a *per se* rule requiring nucleotide-by-nucleotide re-analysis when the structure of the component DNA segments is already known, or readily determined by known procedures. The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed.

See *Enzo Biochem*, 296 F.3d at 1330 (the written description requirement "is the *quid pro quo* of the patent system; the public must receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reiffin v. Microsoft Corp.*, 214 F.3d 1342, 1345-46 (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker*, 559 F.2d 588, 592 n.4 (CCPA 1977) (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

For the chimeric genes of the Capon and Eshhar inventions, the law must take cognizance of the scientific facts. The Board erred in refusing to consider the state of the scientific knowledge, as explained by both parties, and in declining to consider the separate scope of each of the claims. None of the cases to which the Board attributes the requirement of total DNA re-analysis, *i.e.*, *Regents v. Lilly*, *Fiers v. Revel*, *Amgen*, or *Enzo Biochem*, require a re-description of what was already known. In *Lilly*, 119 F.3d at 1567, the cDNA for human insulin had never been characterized. Similarly in *Fiers*, 984 F.2d at 1171, much of the DNA sought to be claimed was of unknown structure, whereby this court viewed the breadth of the claims as embracing a "wish" or research "plan." In *Amgen*, 927 F.2d at 1206, the court explained that a novel gene was not adequately characterized by its biological function alone because such a description would represent a mere "wish to know the identity" of the novel material. In *Enzo Biochem*, 296 F.3d at 1326, this court reaffirmed that deposit of a physical sample may replace words when description is beyond present scientific capability. In *Amgen Inc. v. Hoechst Marion Roussel, Inc.*, 314 F.3d 1313, 1332 (Fed. Cir. 2003) the court explained further that the written description requirement may be satisfied "if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure." These evolving principles were applied in *Noelle v. Lederman*, 355 F.3d 1343, 1349 (Fed. Cir. 2004), where the court affirmed that the human antibody there at issue was not adequately described by the structure and function of the mouse antigen; and in *University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 925-26 (Fed. Cir. 2004), where the court affirmed that the description of the COX-2 enzyme did not serve to describe unknown compounds capable of selectively inhibiting the enzyme.

> The "written description" requirement must be applied in the context of the particular invention and the state of the knowledge. The Board's rule that the nucleotide sequences of the chimeric genes must be fully presented, although the nucleotide sequences of the component DNA are known, is an inappropriate generalization. When the prior art includes the nucleotide information, precedent does not set a *per se* rule that the information must be determined afresh. Both parties state that a person experienced in the field of this invention would know that these known DNA segments would retain their DNA sequences when linked by known methods. Both parties explain that their invention is not in discovering which DNA segments are related to the immune response, for that is in the prior art, but in the novel combination of the DNA segments to achieve a novel result.
>
> The "written description" requirement states that the patentee must describe the invention; it does not state that every invention must be described in the same way. As each field evolves, the balance also evolves between what is known and what is added by each inventive contribution. Both Eshhar and Capon explain that this invention does not concern the discovery of gene function or structure, as in Lilly. The chimeric genes here at issue are prepared from known DNA sequences of known function. The Board's requirement that these sequences must be analyzed and reported in the specification does not add descriptive substance. The Board erred in holding that the specifications do not meet the written description requirement because they do not reiterate the structure or formula or chemical name for the nucleotide sequences of the claimed chimeric genes.

The *Capon* case has very recently been followed by the Federal Circuit, in *Falkner v. Inglis*, App. No. 05-1324, decided May 26, 2006 (Fed. Cir. 2006) (copy enclosed). In a section of the opinion entitled "Recitation of Known Structure Is Not Required" the *Falkner* court, following the *Capon* decision, stated:

> Indeed, a requirement that patentees recite known DNA structures, if one existed, would serve no goal of the written description requirement. It would neither enforce the quid pro quo between the patentee and the public by forcing the disclosure of new information, nor would it be necessary to demonstrate to a person of ordinary skill in the art that the patentee was in possession of the claimed invention. ... Accordingly, we hold that where, as in this case, accessible literature sources clearly provided, as of the relevant date, genes and their nucleotide sequences (here "essential genes"), satisfaction of the written description requirement does not require either the recitation or incorporation by reference (where permitted) of such genes and sequences.

Id. at page 17-18. While *Falkner* dealt with nucleotide and gene sequences, as admitted by the Examiner in the final Office Action such is applicable here as well.

Thus, it is submitted that there is clearly no *prima facie* basis for subject written description rejection, and thus the Board is requested to reverse the Examiner on this basis.

2) Enablement

The Action next rejects the same claims as lacking enablement, again reciting that the specification is enabling only for the same claims, albeit limited to antibody ZME-018 conjugated to TNF for treating melanoma.

In response, Appellants submit that the Examiner has failed to carry her burden of presenting a *prima facie* case of non-enablement and thus has failed to shift the burden to Appellants to come forward with countervailing evidence. Indeed, Appellants have been unable to identify any evidence of non-enablement presented by the Examiner, and have also been unable to identify any cogent reasoning as to why the enablement of the antibody should be questioned.

As noted in MPEP section 2164.04, a patent specification must be taken as in compliance with the enablement requirement unless there is reason to doubt the objective truth of what is stated in the invention disclosure. *In re Marzocchi*, 499 F.2d 220 (CCPA 1971). In particular, to make out a prima facie case the rejection must set forth "why" it doubts the objective truth, and support that "why" with specific evidence, either in the form of documentary evidence or scientifically acceptable reasoning. *Id.* at 224; MPEP 2164.04. In the present case neither has been done.

The Examiner's reasoning is built almost entirely on the erroneous position that animal data is somehow "required" for enablement. This is incorrect. See, *e.g.*, *In re Brana*, 51 F.3d 1560, 1566 (Fed. Cir. 1995). Thus, the mere absence of animal data for all cancers or all

antibody constructs is not a basis for finding *prima facie* non-enablment of claims of a broader scope. The rejection is also based on the Examiner's faulty and unsupported reasoning found in the one full paragraph, page 14 of the Office Action. The allegations appearing in this paragraph, for example, that "those of skill in the art recognize that" ... "clinical correlations are generally lacking" with respect to *in vitro* assays, etc., are unsupported, conclusory and appear to be based on the Examiner's own personal knowledge. Thus, absent an affidavit by the Examiner to attest to these "facts," they must be disregarded. See 37 C.F.R. §1.104(d)(2). Indeed, the only documented "evidence" presented by the Examiner are the articles of Kirkwood *et al.* and Krizen *et al.* (Exhibits 6 and 7 hereto). However, rather than presenting evidence of non-enablement, these two articles merely stand for the proposition that ZME-018 ***is*** adequately enabled; they appear to say nothing that supports an allegation of non-enablement, and the Examiner has pointed us to none.

For the foregoing reasons, the Board is requested to overturn the Examiner's holding of non-enablment.

B. Section 103 Rejections

1) Rejection of claims 7, 26-29 and 32 as unpatentable under 35 U.S.C. §103 over Oldham (Exhibit 1) in view of Freeman (Exhibit 2).

Appellants respectfully traverse this rejection. According to the Examiner, Oldham teaches that "[p]atients were identified as having tumors that reacted with antibody 9.2.27 in vitro and were treated with antibody 9.2.27, resulting in a decrease in melanoma skin lesions, lymph nodes or visceral metastasis," citing pages 2802, col. 2 and 2804, col. 2, for this proposition. We submit that the Examiner's recitation of what Oldham teaches in this regard is in error. At the bottom of column 2, page 2804, Oldham states that "[t]here were ***no*** measurable

decreases in the size of melanoma skin lesions, lymph nodes, or visceral metastases during treatment with 9.2.27" (emphasis ours). So, the reference says the exact opposite as compared to the representations of the Examiner. Furthermore, even though the particular tumors targeted by 9.2.27 were apparently tested to determine that they had the targeted antigen, it appears that no such testing was carried out with respect to the other antibody investigated by Oldham, the T101 antibody.

For the foregoing reasons, there is accordingly no general teaching in Oldham to pre-test patients as a means of selecting the appropriate antibody, and no teaching that 9.2.27 is in any way a useful antibody that should be incorporated into an immunotoxin. As such, there is simply no motivation in Oldham to use the 9.2.27 antibodies as a fusion partner for making immunotoxins. This is important distinction in that Oldham may well suggest to use "some" antibodies in the preparation of immunotoxins – the Appellants do not presently dispute that immunotoxins *per se* were well known as of the filing date – however, Oldham cannot be said to suggest using 9.2.27 in an immunotoxin. Furthermore, what is particularly disputed is the Examiner's conclusion that the *pre-testing* of patients as a means of selecting an appropriate immunotoxin is obvious in light of Oldham. Such a conclusion is not supported by the evidence of record, which does ***not*** teach or suggest using antigen pre-testing as a clinical subpopulation selection scheme – at best, Oldham simply states that for the purposes of this small clinical trail the patients tumors were analyzed.

The secondary of Freeman, as far as we can determine, also fails to in any way teach or suggest the concept of pre-screening cancer patients to be treated to characterize the antigenic susceptibility of the cancer to a particular immunotoxin. Thus, even though Freeman is actually

a review article and written well after Oldham, it provides no hint of the presently claimed invention.

2) Rejection of claims 13, 14 and 16 as unpatentable under 35 U.S.C. §103 over Oldham in view of Freeman, further in view of Ferris (Exhibit 3) and Bregman (Exhibit 4)

Claims 13, 14 and 15 are directed specifically to targeting cytokines such as TNF, and the Examiner cites Ferris and Bergman for this proposition.

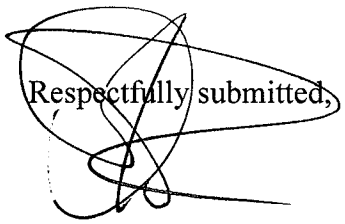
In response, Appellants would agree that the use of TNF as a biological response modifier for use in connection with immunotoxins was known as of the Appellant's filing date. However, Appellants have been unable to identify any teaching in either of these references that is relevant to pre-screening of tumors and selection of antibodies on this basis, prior to clinical administration.

3) Rejection of claim 23 as unpatentable under 35 U.S.C. §103 over Oldham and Freeman in view of Anderson (Exhibit 5)

Claim 23 is a "fusion" claim, against which the Examiner has now cited Anderson. In response, Appellants would agree that the use of the "fusion" concept in connection with immunotoxin preparation was known as of the Appellant's filing date. However, Appellants have been unable to identify any teaching in Anderson that is relevant to pre-screening of tumors and selection of antibodies on this basis, prior to clinical administration.

VIII. CONCLUSION

Appellants believe that the foregoing remarks fully respond to all outstanding matters for this application. Appellants respectfully request that the Board reverse the rejections of all claims.



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Date: January 11, 2007

I. CLAIMS APPENDIX

1. – 6. (Canceled)

7. (Previously presented) The method of claim 26, wherein said cancer is selected from the group consisting of breast cancer, cervical carcinoma and melanoma.

8. – 9. (Canceled)

10. (Previously presented) The method of claim 7, wherein the patient has been diagnosed with cancer and cells of the cancer express an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009), and further wherein the protein is a monoclonal antibody that recognizes and binds the antigen.

11. – 12. (Canceled)

13. (Previously presented) The method of claim 24, wherein the biological response modifier is a cytokine.

14. (Previously Presented) The method of claim 13, wherein the cytokine is TNF.

15. (Withdrawn) The method of claim 14, wherein the TNF is TNF-beta.

16. (Previously Presented) The method of claim 14, wherein the TNF is TNF-alpha.

17. (Withdrawn) The method of claim 13, wherein the cytokine is an interleukin.

18. (Withdrawn) The method of claim 17, wherein the interleukin is interleukin-1 or interleukin-6.

19. (Withdrawn) The method of claim 13 wherein the cytokine is an interferon.

20. (Canceled)

21. (Previously presented) The method of claim 24, wherein the protein's antigen recognition site recognizes and binds to the ZME-018 antigen, an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009).

22. (Canceled)

23. (Currently amended) The method of ~~claim 24~~ claim 26, wherein the protein with an antigen recognition site is fused to the biological response modifier.

24. (Currently amended) The method of ~~claim 24~~ claim 26, wherein the protein with an antigen recognition site is conjugated to the biological response modifier.

25. (Previously presented) The method of claim 14, wherein the protein's antigen recognition site recognizes and binds to the ZME-018 antigen, an antigen recognized by monoclonal antibody ZME-018 (ATCC accession number HB 11009).

26. (Previously presented) A method of treating cancer in a human patient in need of such treatment, the method comprising the steps of:

(a) identifying a patient having a tumor, which tumor comprises cells for targeting and wherein those cells comprise a cell surface antigenic marker at concentrations in excess of that found at other non-target sites;

(b) obtaining a composition comprising a protein with an antigen recognition site directed toward a cell surface associated antigen conjugated or fused to the biological response modifier, wherein it has been determined that cells of the patient's cancer express an antigen recognized and bound by the protein with an antigen recognition site; and

(c) administering an amount of the composition to the patient effective to treat the cancer.

27. (Previously presented) The method of claim 26, wherein the patient is diagnosed as having a tumor with a specific antigenic determinant that will allow targeting and concentration of the biological response modifier at the site where it is needed to kill tumor cells.

28. (Previously presented) The method of claim 26, wherein the protein is an antibody.

29. (Previously presented) The method of claim 28, wherein the antibody is a monoclonal antibody.

30. (Previously presented) The method of claim 7, wherein the cancer is breast cancer.

31. (Previously presented) The method of claim 7, wherein the cancer is cervical carcinoma.

32. (Previously presented) The method of claim 7, wherein the cancer is melanoma.

II. EVIDENCE APPENDIX

Exhibit 1 – Oldham *et al.*, made of record in the Office Action dated 7/18/06

Exhibit 2 – Freeman *et al.*, made of record in the Office Action dated 7/18/06

Exhibit 3 – Ferris *et al.*, made of record in the Office Action dated 7/18/06

Exhibit 4 – Bregman *et al.*, made of record in the Office Action dated 7/18/06

Exhibit 5 – Anderson *et al.*, made of record in the Office Action dated 7/18/06

Exhibit 6 -- Kirkwood *et al.* made of record in the Office Action dated 7/18/06

Exhibit 7 – Krizen *et al.* made of record in the Office Action dated 7/18/06

EXHIBIT 1

Lymphokines, Monoclonal Antibodies, and Other Biological Response Modifiers in the Treatment of Cancer

ROBERT K. OLDHAM, MD, GARY B. THURMAN, PhD, JAMES E. TALMADGE, PhD, HENRY C. STEVENSON, MD, AND KENNETH A. FOON, MD

Biologicals and biological response modifiers (BRMs) represent a new class of agents for cancer therapy. Historically, there have been many attempts to stimulate the immune response with nonspecific immunomodulators in the form of bacterial extracts, viruses, and chemicals. Although these approaches have occasionally proven useful under defined conditions in experimental models, their extension to the clinic has been largely unsuccessful. Recent advances in molecular biology and hybridoma technology have made available genetically engineered lymphokines and cytokines, as well as monoclonal antibodies, as highly purified biologicals for cancer treatment. These agents may act directly on tumor cells and/or may act on the patient's own biological responses to induce an antitumor response. Selective defects in T-cell function have recently been identified in cancer patients and in patients with acquired immunodeficiency syndrome (AIDS). Simultaneously, the availability of gamma interferon (γ-IF) and interleukin-2 (IL-2) may allow for the selective correction of these T-cell deficits, leading to restoration of the patient's immune responses and perhaps correction of the clinical syndromes. Preliminary data suggest that γ-IF and IL-2 have *in vitro* activity on these T-cell defects, and the preliminary evidence that these agents have activity *in vivo* will be reviewed. Extensive trials are being conducted at the National Cancer Institute with monoclonal antibodies as anticancer agents. Animal model experiments have demonstrated considerable antitumor activity of immunoconjugates using monoclonal antibodies tied to toxins. Preliminary clinical results suggest that T-101 in leukemia and lymphoma and 9.2.27 in malignant melanoma may prove useful as specific reagents in the treatment of these disorders. While the antitumor effects with these antibodies have not been dramatic, our preliminary data in approximately 30 patients with leukemia, lymphoma, and melanoma clearly demonstrate the ability of intravenous monoclonal antibody to locate and specifically label tumor cells bearing the target antigens. It has been possible to localize antibody on the tumor cells in melanoma deposits that are barely visible in the skin. These data and radioimaging data suggest a future role for immunoconjugates as anticancer agents.

Cancer 54:2795–2806, 1984.

THE USE of chemical and biological compounds to modulate biological responses has been an area of investigation over the last 20 years. Although a variety of chemicals, bacterial extracts, and viruses have been found to modulate immune responses in experimental animals and, to a more limited extent in man, these "nonspecific" immunomodulators, when used in clinical trials, have not been highly effective. With the advent of "genetic engineering," the techniques of molecular biology have been applied to the isolation of genes and their subsequent translation into appropriate host production systems, which now allow for the virtually unlimited production of highly purified biological compounds for experimental and therapeutic use. As a result of this progress in molecular biology, several new biologicals are being evaluated in preclinical models and are being entered into clinical trials (Table 1).[1]

The continued investigation of nonspecific immunomodulators as well as the recent advent of genetically engineered biologicals makes the need for predictive preclinical assays of biological activity and efficacy apparent. *In vitro* assays of biological activity (bioassays) are generally used to define and quantitate the activity of a given biological substance. Subsequently, radioimmunoassays or other methods of determining the number of molecules available allow for the precise determination of levels of these biologicals in appropriate fluids. Finally,

Presented at the American Cancer Society National Conference: Advances in Cancer Therapy, December 8–10, 1983, New York, New York.

From the Biological Response Modifiers Program, National Cancer Institute-Frederick Cancer Research Facility, Frederick, Maryland.

Address for reprints: Robert K. Oldham, MD, Biological Therapy Institute, Riverside Drive, Franklin, TN 37064.

Accepted for publication March 12, 1984.

TABLE 1. Biologicals and Biological Response Modifiers

Immunomodulators (Chemicals, bacterial extracts, viruses, *etc.*)
Lymphokines/cytokines
Growth/maturation factors
Effector cells
Tumor-associated antigens
Monoclonal antibodies

there is the need to assess the *in vivo* activity of these materials in preclinical models to develop predictive assays for clinical efficacy and provide information useful in the rational design of clinical trials.

The strategy and design of the preclinical screen within the Biological Response Modifiers Program (BRMP) has been described.[2] Preliminary results with several immunomodulators assessed in this screening program have been reported.[3-7] The evaluation of biologicals such as interferon and the interleukins has been initiated in this screening process. For biologicals, there is often the complicating problem of species specificity. Not all lymphokines/cytokines of human origin are active in rodent systems. For example, most of the naturally occurring human interferons (alpha, beta, and gamma) have little activity on rodent cells, making the preclinical assessment of these biologicals in our screening systems of little use. For those biologicals such as interleukin-2 that do have activity on rodent cells, this preclinical system may be of more value.

While lymphokines and cytokines have activity on host biological responses, they may also have some direct activity on the tumor cells. This is best exemplified by the direct antiproliferative activity of the interferons. By contrast, monoclonal antibodies are the prototypic substances to be evaluated for specific, direct antitumor activity. Monoclonal antibodies can be designed to act as immunomodulators and affect various components of the immune system, but their early evaluation has centered on their use as specific targeting agents in cancer treatment.[8] We have initiated a series of studies with antibodies directed at the 65-kd antigen on normal T-cells and on certain human leukemias and lymphomas.[9] Clinical trials have also been initiated using an antimelanoma monoclonal antibody.[10] It is apparent from these studies that monoclonal antibody can be given intravenously and can be expected, if given in sufficient amounts and in appropriate schedules, to reach the tumor cells variously distributed in the patient. Our preliminary results using the monoclonal antibodies in clinical trials will be described.

Preclinical Screening of Biologicals and Biological Response Modifiers

The preclinical screening system established by the BRMP for the assessment of biologicals and biological response modifiers was designed to specifically evaluate the effects of these agents *in vitro* and *in vivo* on T- and B-cells, macrophages, and natural killer (NK)-cells. As

TABLE 2. Preclinical Screening of Biological Response Modifiers

Progression	Macrophages	NK-Cells	T-Cells	B-Cells
In vitro activation, *in vitro* testing	*In vitro* activation for 24-hr, 72-hr cytotoxicity assay of [^{125}I]IUdR tumor cells and 18-hr [^{111}In] tumorcells	*In vitro* activation for 24-hr, 4-hr cytotoxicity assay α[^{51}Cr]-YAC	MLR allogeneic MLTR-CMC allogeneic	
In vivo activation, *in vitro* testing	Inject BRM IV; harvest AM and test for tumoricidal activity	Inject BRM IV; measure NK activity *in vitro;* IF stimulation	Immunization αTSTA CTL	Stimulation of specific antibody production
In vitro activation, *in vivo* testing		Prevention of metastasis in 3-wk-old mice (fibrosarcoma)	Immunization αTSTA, SC challenge; alteration of tumor growth in UV-irradiated mice after immunization	
Mechanism	Kinetics of activation; induction of hyporesponsiveness	Kinetics of activation; induction of hyporesponsiveness		

NK: natural killer; IUdR: iododeoxyuridine; MLR: mixed lymphocyte reaction; CMC: cell mediated by toxicity; BRM: biologic response modifiers; IV: intravenously; AM: alveolar macrophages; IF: interferon; TSTA: tumor-specific transplantation antigens; CTL: cytotoxic lymphocytes; SC: subcutaneously; UV: ultraviolet light.

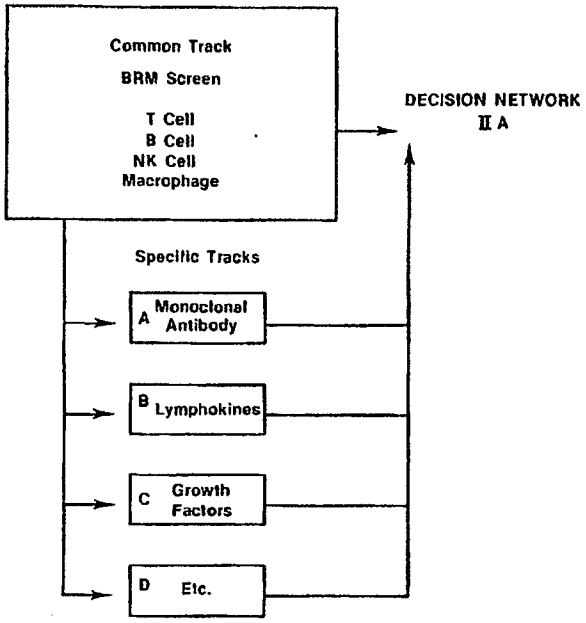

FIG. 1. The BRMP screening program consists of a common track and specific tracks. The common track is in place and evaluating immunomodulators and selected lymphokines and cytokines. The specific tracks still are being formulated.

is illustrated in Table 2, the common track screen is designed to evaluate these agents on the various cellular and humoral components of the immune response. In addition, for the evaluation of agents and approaches with more selective targets, specific tracks are being considered for possible inclusion in the screen (Fig. 1). We have evaluated more than 15 compounds in the screening program and, as is illustrated in Table 3, immunomodulators have selective activities on certain elements of the immune response. For example, the thymosins have been most active in T-cell assays but do not enhance NK-cell or macrophage activity, whereas MVE-2 was more stimulatory for NK-cells and macrophages. By contrast, MVE-2 stimulated macrophages and enhanced NK-cell activity when given intravenously but did not enhance NK-cell activity when added *in vitro*. This may be explained by MVE-2 stimulated interferon release from macrophages, which secondarily activates NK-cells *in vivo*. Poly ICLC was quite efficacious in stimulating macrophages and NK-cells both *in vitro* and *in vivo*, whereas MDP was selective for T-cells and macrophages but had little stimulatory affects on NK-cells. These data illustrate that immunomodulators selectively effect certain components of the immune system and some may function *in vitro* but have little activity *in vivo*. By contrast, others may be effective *in vivo* but

TABLE 3. Biological Response Modifiers Program Screening Summary

Assay of immunomodulation	Thymosin F5	MVE-2	Poly ICLC	Nor-MDP
T-cell blastogenesis	+	−	−	+
Allogeneic MLR stimulation	+	−	−	+
Allogenic MLTR-CMC activation	+	−	−	+
Syngeneic MLTR-CMC activation	+	NT	NT	NT
Immunoadjuvant for specific CTL	+	+	+	+
Immunoadjuvant for tumor challenge	+	+	+	+
In vitro NK cell activation	−	+	+	−
In vivo NK cell activation	−	−	+	−
In vivo NK cell immunoprophylaxis	−	+	+	−
In vitro macrophage activation	−	+	+	+
In vivo macrophage activation	−	+	+	+
Interferon stimulation	−	+	+	NT

Thymosin F5: an extract of calf thymus polypeptides provided by Hoffmann La Roche, Inc., Nutley, New Jersey (optimal dose, 12.5 mg/kg); MVE-2; maleic anhydride-divinyl ether, obtained from Dr. Richard Carreno, Adria Laboratories, Columbus, Ohio (optimal therapeutic dose, 25 mg/kg); Poly ICLC: poly inosine-polycysteine stabilized with poly L-lysine and carboxymethyl cellulose provided by Dr. Hilton Levy, National Institute of Allergy and Infectious Diseases, Frederick, Maryland (optimal dose, 0.5 mg/kg); Nor-MDP; Nor-muramyl dipeptide, an analogue of MDP, which is the minimally active subunit from BCG, obtained from Ciba-Geigy, Summit, New Jersey (optimal therapeutic dose, 75 mg/kg); MLR: mixed lymphocyte reaction; MLTR: mixed lymphocyte tumor cell reaction; CMC: cell-mediated toxicity; NT: not tested; CTL: cytotoxic lymphocytes; NK: natural killer.

have little activity *in vitro*, particularly when they undergo metabolic activation or alternation. In addition to the *in vitro* testing of these immunomodulators and the testing *in vitro* after *in vivo* injection, the screening process also evaluates the *in vivo* antitumor activity of these compounds. As is illustrated in Table 4, we have

TABLE 4. Preclinical Screening of Biological Response Modifiers: Tumor Therapy Models

Treatment of metastases	Treatment of autochtonous tumors
Experimental metastases survival lung colony counts	UV-induced skin tumors Intralesional Twice weekly IV for 3 wk
Spontaneous metastases 12 wk lung colony counts	NMU-induced mammary tumors Twice weekly IV for 3 wk
Lifetime survival after experimental and spontaneous metastasis	

UV-ultraviolet light; IV: intravenously; NMU: nitrosomethylurea.

TABLE 5. Comparison of Biological-Response-Modifier-Induced Immunotherapy

	MVE-2	OK-432	Poly ICLC	MTP-PE	A/D BgL IFN	Thymosin F5	Lentanin	NED-137	Nor-MDP
Nonspecific immunoprophylaxis*	+	+	+	STA	+	–	–	–	–
Specific immunoprophylaxis†	+	+	+	+	+	+	STA	–	+
Immunotherapy of experimental metastases‡	Prolongation of survival	STA	+	+	+	+	STA	–	+
Immunotherapy of spontaneous metastases§	–	–	+	+	STA	+	–	–	+

* The biological response modifier (BRM) was injected intravenously (IV), 48 hr prior to the IV injection of 50,000 syngenic tumor cells (UV-2237 mm). Necropsy was performed on the mice 25 days following tumor challenge and the extent of pulmonary tumor burden was quantitated.

† Mice were immunized with a suboptimal tumor vaccine consisting of 10^6 irradiated tumor cells obtained by the collagenase-DNAase dissociation of subcutaneous tumors. The BRM was either injected alone or admixed with the tumor vaccine to test its adjuvant activity. Tumor challenge was 10 days following vaccination and consisted of the footpad injection of 10^5 tumor cells. Tumor growth and volume were determined weekly.

‡ Mice were injected iv with 50,000 B16-BL-6 tumor cells and 72 hours later therapy was initiated. Therapy consists of the twice weekly injection of the BRM for 4 weeks.

§ Mice received footpad injection of 50,000 B16-BL-6 tumor cells. When the tumors reached an average diameter of 1 cm, the tumor-bearing limb was resected at mid femur to include the popliteal lymph node. Therapy was initiated 72 hr later and consisted of twice weekly injections for 4 weeks. Mice that remain alive 4 wk following the death of the last saline control animal underwent necropsy to confirm their tumor-free status.

MVE-2. maleic anhydride-divinyl ether, obtained from Dr. Richard Carraro, Adria Laboratories, Columbus, Ohio (optimal therapeutic dose, 25 mg/kg); OK-432: a penicillin killed-lyophilized preparation of a virulent strain of group A *Streptococcus pyogenes*, provided by the Chugai Pharmaceutical Co., Ltd., Tokyo, Japan (optimal therapeutic dose, 1 KE/animal); Poly ICLC: poly inosine-polycysteine stabilized with poly L-lysine and carboxymethyl cellulose, provided by Dr. Hilton Levy, National Institute of Allergy and Infectious Diseases, Frederick, Maryland (optimal dose, 0.5 m/kg); MTP-PE: muramyl tripeptide-phosphatidyl ethanolamine, which was incorporated into multilamillar vesicles composed of phosphatidyl-serine at a 7:3 molar ratio. The MTP-PE was obtained from Ciba Geigy, Summit, New Jersey (optimal therapeutic dose, liposomes, 2.5 μmol phospholipid incorporating 12 μg of MTP-PE); A/D BgL II IFN; a hybrid recombinant interferon obtained from Hoffmann La Roche, Inc., Nutley, New Jersey (optimal therapeutic dose, A/D BgL II IFN, 10,000–50,000 U/animal); Thymosin F5: a polypeptide extract of calf thymus provided by Hoffmann La Roche, Inc., Nutley, New Jersey (optimal dose, 12.5 mg/kg); Lentinan: an aqueous extract from the *Lentinus edodes* mushroom provided by Ajinomoto Co., Inc., Tokyo, Japan (highest dose tested therapeutically, 50 mg/kg); NED-137: a combination of a number of small polymeric compounds with a mean molecular weight of 800, which was obtained from Monsanto Co., St. Louis, Missouri. (highest dose tested therapeutically, 50 mg/kg); Nor-MDP: Nor-muramyl dipeptide, an analogue of MDP, which is the minimally active subunit from bacillus Calmelte-Guerin, obtained from Ciba Geigy, Summit, New Jersey (optimal therapeutic dose, 75 mg/kg); STA: slight therapeutic activity is defined as a statistically significant decrease in metastatic tumor burden or prolongation of survival that was significantly less efficacious than the positive control.

relied on those transplantation models that emphasize the evaluation of metastasis and on models of primary autochthonous tumors using ultraviolet (UV) light and nitrosomethylurea for tumor induction. This emphasis on metastasis and on primary tumors was selected anticipating that these preclinical *in vivo* models would be more predictive for clinical activity.[2] As is shown in Table 5, several immunomodulators have activity in the inhibition of experimental and spontaneous metastasis. In addition, we now have evidence for the inhibition of autochthonous tumor growth by liposome-encapsulated muramyltripeptide and by poly ICLC (Fig. 2 and Table 6).

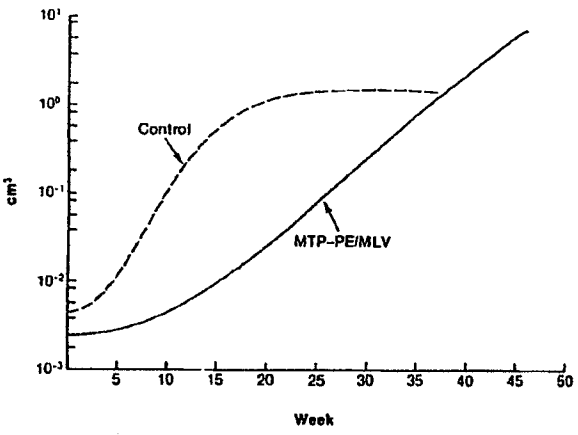

FIG. 2. The size of UV-induced ear tumors of control animals (treated with saline) is contrasted with the retardation of growth seen when muramyltripeptide in liposomes is given intravenously to animals with UV-induced tumors.

These results are encouraging, but they also illustrate the complexity of preclinical evaluations. The ultimate assessment of the screening program must require the translation of multiple immunomodulating agents from these preclinical trials into clinical trials to determine whether these assays are truly predictive for clinical activity in humans. Limited correlations are currently being done for MVE-2, the thymosins, and the interferons and may soon be possible for the interleukins.

Lymphokines and Cytokines

Mammalian cells produce a huge number of lymphokines and cytokines in response to various stimuli. As

is illustrated for interferon induction by Figure 3, cells may produce one or many biologicals when properly induced, and these biologicals may each have a complex series of actions on the individual's biological responses. The macrophage is a virtual factory for the production of biologically active factors (Table 7). Some lymphokines and cytokines appear to be restricted by the major histocompatibility complex (MHC) and these may be characterized as specific or nonspecific (Table 8). The majority of lymphokines and cytokines are not known to be restricted by the MHC and are not antigen-specific. There are over 100 such substances that have been described by name, based on observed biological activity (Table 9). The substances can be isolated from the products of normal cells, normal cell lines derived from normal tissues, or tumor-derived cell lines. The actual number of lymphokines and cytokines remains to be defined by careful genetic analysis; this will be possible through the production of purified genetically engineered biologicals. It is expected that a large number of these biologicals will be cloned and available in sufficient quantities for preclinical and clinical evaluation. This probability makes even more apparent the need for predictive assays of clinical efficacy so that resources can be directed toward those biologicals that are most likely to be active in clinical trials. Obviously, the use of these biologicals in therapeutics is not restricted to cancer therapy, as many of these agents and approaches may be quite useful for autoimmune, inflammatory, and even infectious disorders.

TABLE 6. Therapy of NMU-Induced Mammary Tumors

	Saline	Poly ICLC	Thymosin F5
Mean tumor size at initiation of therapy (cm^3)	0.86 ± 0.97	1.14 ± 1.1	1.29 ± 1.4
Mean tumor size at evaluation (cm^3)	12.11 ± 18.64	5.13 ± 5.69	8.26 ± 1.3
Percent tumor free at evaluation	0%	30%	15%
Percent of animals with multiple tumors	85%	45%	70%
Percent survival at evaluation	90%	95%	90%
Mean time since initiation of therapy (wk)	10.5 ± 2.3	10.5 ± 1.9	10.6 ± 2.0

A single dose of N-nitroso-N-methylurea (50 mg/kg) was administered intravenously to 50-day-old female Sprague-Dawley rats. Biological response modifier (BRM) therapy was initiated when the first lesion was noted and consisted of twice-a-week injection of the agent for 4 weeks. The BRMs included Poly ICLC at 0.75 mg/kg and thymosin F5 at 5 mg/kg with saline controls. Tumor diameters were assessed twice a week and their volume calculated using the formula for a spheroid. The occurrence of satellite tumors is noted and reported as multiple tumors (N = 20).

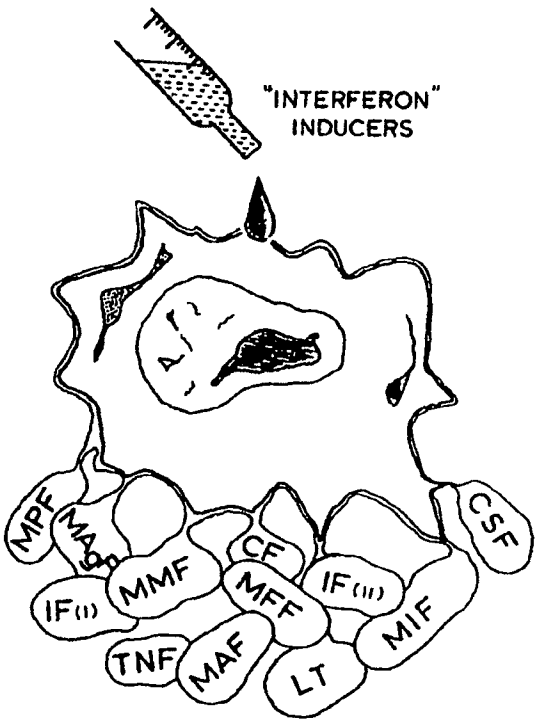

FIG. 3. Induction of various lymphokines and cytokines with stimulation by an interferon inducer.

Gamma Interferon

Perhaps the first cloned biological available for the clinic that may act as an interleukin will be gamma interferon. Extensive testing of the alpha interferons and early testing of the beta interferons have emphasized their antiviral activity and their potential antiproliferative activity in humans. Both of these interferons also have immunomodulatory effects, and these effects are analyzed concomitantly. However, preclinical studies seem to indicate that gamma interferon is a more diverse and powerful immunomodulator than alpha or beta interferon, and there are already indications that it may be responsible for some of the biological activities previously ascribed to other lymphokines and cytokines.[11–15] Cloned gamma interferon is currently entering Phase I clinical trials; it will be of great interest to determine which biological responses are reproducibly altered by its administration. We have conducted a trial of partially purified natural gamma interferon in humans.[16] The information from this trial suggests that the intravenous route of administration is necessary to achieve antiviral titers in the serum. In addition, prolonged infusion may be valuable to maintain the circulating gamma interferon activity. Toxicities have included fever, chills, fatigue,

TABLE 7. Secretory Products of Mononuclear Phagocytes

- Enzymes
  - Lysozyme
  - Neutral proteases
    - Plasminogen activator
    - Collagenase
    - Elastase
    - Angiotensin-convertase
  - Acid hydrolases
    - Proteases
    - Lipases
    - (Deoxy) ribonucleases
    - Phosphatases
    - Glycosidases
    - Sulfatases
  - Arginase
- Complement components
  - C1
  - Factor B
  - Factor D
  - Properdin
  - C3b inactivator
  - β1H
- Enzyme inhibitors
  - Plasmin inhibitors
  - α2-Macroglobulin
- Binding proteins
  - Transferrin
  - Transcobalamin II
  - Fibronectin
- Nucleosides and metabolities
- Endogenous pyrogens (IL-1, *etc.*)
- Reactive metabolites of oxygen
- Bioactive lipids
  - Arachidonate metabolites
  - Prostaglandin E_2
  - 6-keto-Prostaglandin $F_{1\alpha}$ (from prostacyclin)
  - Thromboxane
  - Leukotrienes
  - Hydroxy-eicosatetraeneoic acids (including slow-reacting substance of anaphylaxis)
  - Platelet-activating factors
- Factor chemotactic for neutrophils
- Factor regulating synthesis of proteins by other cells
- Factors promoting replication of
  - Lymphocytes (lymphocyte activating factors-IL-1)
  - Myeloid precursors (colony-stimulating factors)
  - Erythroid precursors
  - Fibroblasts
  - Microvasculature
- Factors inhibiting replication of
  - Lymphocytes
  - Tumor cells
  - Viruses (interferon)
  - *Listeria monocytogenes*

Adapted from Nathan CF, Murray HW, Cohn ZA. Current concepts: The macrophage as an effector cell. *N Engl J Med* 1980; 303:622–626.

TABLE 8. Mediators Restricted by MHC

Antigen-specific mediators	Antigen-nonspecific mediator
Helper Factors	Helper Factors
GRF (genetically restricted factor)	AEF (allogeneic effect factor)
"IgT"	Specific autostimulatory factor
Helper factor for IgE	Complement components
Antigen-specific T-cell factor	Suppressor Factors
Antigen-specific TRF	MLR-suppressor factor
TF (transfer factor)	Low-molecular-weight Ia
Suppressor Factors	Complement components
"IgT"	
Antigen-specific suppressive T-cell factor	
IgE suppressor factor	
Allotype-suppression factor	
Soluble suppressor of contact sensitivity	
Immunosuppressive factor (GAT)	
Tumor-specific blocking factor	
Factors Acting on Macrophages	
SMAF (specific-macrophage-arming factor	
Antigen-dependent MIF	

Adapted from Waksman BH. Overview: Biology of the lymphokines. In: Biology of Lymphokines. New York: Academic Press, 1979; 585–616.

MHC: major histocompatibility index; TRF: T-cell-replacing factor; MIF: macrophage inhibitory factor; MLR: mixed lymphocyte reaction.

and anorexia similar to that seen with alpha and beta interferon. In addition, dose-limiting hypotension was apparent in our trial at 60 million U/m[16]. When compared with our previous studies with alpha interferon, antiviral titers were generally lower and more transiently maintained with gamma interferon. In these early studies, significant immunomodulation of NK-cell and monocyte activity was not apparent. An antiproliferative effect on lymphocytes was seen, but no antitumor activity was observed in this initial trial. Considerable lot-to-lot variation in the biological activity was noted; this observation emphasizes the need to pursue biological pharmaceuticals of high purity in the absence of other contaminating substances in order to understand the biological effects of a particular lymphokine or cytokine.

Recent studies on gamma interferon have indicated that it has both macrophage inhibitory factor (MIF) and macrophage activity factor (MAF) activity.[11,12] The *in vitro* use of cloned gamma interferon and of monoclonal antibody to gamma interferon has proven to be a powerful approach in determining the precise *in vitro* activities associated with this molecule. It is clear from these studies that antibody that neutralizes the antiviral activity of gamma interferon can diminish both the MIF and MAF activity of cloned gamma interferon. By contrast, when cell-line or stimulated-cell supernatants are used as a source for MIF or MAF, the antibody does not always eliminate these activities. This seems to indicate that gamma interferon may have MIF and MAF activity, but there are other substances produced by mammalian cells with these activities as well.

The broader immunomodulatory activity of gamma interferon and the reported higher antiproliferative activity of this interferon in comparison to alpha and beta interferon is of interest. In addition, recent reports have emphasized the synergistic capability of alpha or beta interferon when added to gamma interferon.[17] All of these preliminary reports will need to be confirmed by more extensive preclinical trials, and the exploitation of these effects in clinical trials is anticipated.

Interleukin-2

Interleukin-2 (IL-2) was originally described as a biological activity of stimulated lymphocytes that supported the growth of certain T-cells bearing the IL-2 receptor.[18] Subsequent generation of IL-2 from stimulated cell lines and, finally, the cloning of IL-2 have made available large quantities of purified material for preclinical and clinical trials.[19] A model for the activity of IL-2 is illustrated in Figure 4, and its biochemical and biological properties are outlined in Table 10. Because of the ability to support T-cell proliferation, IL-2 has been evaluated in two general approaches: (1) IL-

TABLE 9. Antigen-Nonspecific Mediators, Unrestricted by MHC

- Helper factors
  - LAF (lymphocyte-activating factor-IL-1)
  - NMF (normal macrophage factor)
  - BAF (B-cell-activating factor)
  - TRF (T-cell-replacing factor)
  - MP (mitogenic protein)
  - TDF (thymus differentiation factor)
  - Transferrin
  - MF (mitogenic [blastogenic] factor)
  - NSF (nonspecific factor)
  - TDEF (T-cell-derived enhancing factor)
  - TEF (thymus extracted factor)
  - Complement components
  - DSRF (deficient-serum-restoring factor)
- Suppressor factors
  - Inhibitor(s) of DNA synthesis
  - AIM (antibody-inhibitory material)
  - IDS (inhibitor of DNA synthesis)
  - LIF (lymphoblastogenesis-inhibition factor)
  - FIF (feedback-inhibition factor)
  - MIFIF (MIF-inhibition factor)
  - SIRS (soluble-immune-response suppressor)
  - LIFT (lymphocyte-inhibiting factor-thymus)
  - IRS (immunoregulatory α-globulin)
  - Chalones
  - IF (interferons)
  - AFP (α-fetoprotein)
  - LDL (low-density lipoproteins)
  - CRP (C-reactive protein)
  - Fibrinogen degradation products
  - NIP (normal immunosuppressive protein)
  - LMWS (low-molecular-weight suppressor)
  - HSF (histamine-induced suppressor factor)
  - TCSF (T-cell-suppressive factor)
- Factors acting on inflammatory cells
  - MIF (migration-inhibitory factor)
  - MCF (macrophage chemotactic factor)
  - MSF (macrophage-slowing factor)
  - MEF (migration-enhancement factor)
  - MAF (macrophage-aggregation factor)
  - MAF (macrophage-activating factor)
  - MFF (macrophage-fusion factor)
  - PRS (pyrogen-releasing substance)
  - LIF (leukocyte-inhibition factor)
  - NCF (neutrophil chemotactic factor)
  - PAR (products of antigenic recognition)
  - BCF (basophil chemotactic factor)
  - ECF (eosinophil chemotactic factor)
  - ESP (eosinophil-stimulation promoter)
  - LCF (lymphocyte chemotactic factor)
  - LTF (lymphocyte-trapping factor)
- Factors acting on vascular endothelium
  - SRF (skin reactive factor)
  - TPF (thymic permeability factor)
  - LNPF (lymph-node-permeability factor)
  - LNAF (lymph-node-activating factor)
  - AIPF (anaphylactoid-inflammation-promoting factor)
  - IVPF (increased vascular permeability factor)
- Factors acting on other cells
  - Interferons
  - TMIF (tumor-cell-migration-inhibition factor)
  - OAF (osteoclast-activating factor)
  - Fibroblast chemotactic factor
  - Pyrogens
  - FAF (fibroblast-activating factor)
- Growth-stimulating factors
  - BCGF (B-cell growth factor)
  - BCDF (B-cell differentiation factor)
  - MGF (macrophage growth factor)
  - MF (mitogenic [blastogenic] factor)
  - LIAF (lymphocyte-induced angiogenesis factor)
  - CSF (colony-stimulating factor)
  - TDF (thymus differentiation factor)
  - Thymopoietin, thymosin
  - TCGF (T-cell growth factor-IL-2)
  - IL-3 (interleukin 3)
  - EGF (epideimal growth factor)
- Direct-acting factors
  - Lysosomal enzymes
  - CTF (cytotoxic factors)
  - MTF or MCF [macrophage toxic [cytotoxic] factor)
  - SMC (specific macrophage cytoxin)
  - MCF (macrophage cytolytic factor)
  - ACT (adherent cell toxin)
  - Chromosomal breakage factors
  - Microbicidal factors
  - LT (lymphotoxin)
  - PIF (proliferation inhibitory factor)
  - CIF (cloning inhibition factor)
  - IDS (inhibitory of DNA synthesis)
  - Transforming factors
  - TNF (tumor necrosis factor)

Adapted from Waksman BH. Overview: Biology of the lymphokines. In: Biology of Lymphokines. New York: Academic Press, 1979; 585–616.

MHC: major histocompatibility index.

2 has been used to support the proliferation of T-effector cells *in vitro* in the hope that large quantities of these effector cells can be used therapeutically after expansion to sufficient numbers.[20,21] This approach has shown efficacy in experimental models, and early-phase clinical trials are underway in an attempt to exploit this growth-promoting capability for T-effector cells. (2) There have been recent attempts to directly infuse IL-2 to stimulate T-cell proliferation *in vivo* and/or to correct a T-cell defect *in vivo*.[21,22] The recently described acquired immunodeficiency syndrome (AIDS) represents a clinical model in which IL-2 may be useful. Patients with this syndrome have a selective defect in T-helper cells and a reversal of the T-helper/suppressor ratio in addition to certain other immunologic deficiencies that may underlie their susceptibility to multiple infections and may also contribute to the development of Kaposi's sarcoma and lymphoma, which is increasingly being seen in these patients. There is now preliminary evidence that IL-2 partially corrects the T-cell defect when the AIDS pa-

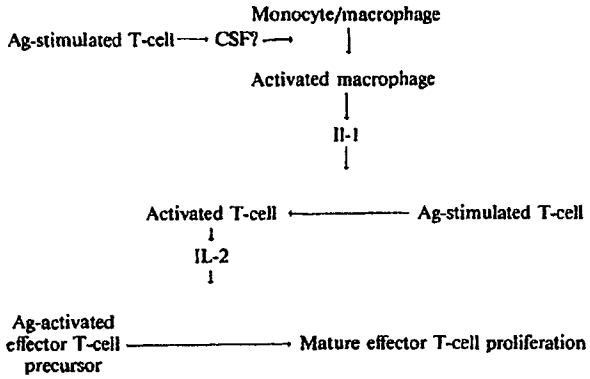

FIG. 4. Proposed model for interleukin stimulation of cellular immune responses.

tient's T-cells are coincubated with this biological *in vitro*.[23] Preliminary clinical trials administering IL-2 to patients with AIDS have also shown some encouraging results with respect to correcting this defect.[24,25] In addition to the effects on T-cells, IL-2 plays a role in supporting the growth of NK-cells and may stimulate the cytotoxic activity.

Monoclonal Antibodies

The therapeutic use of murine-derived monoclonal antibodies in early Phase I trials in humans have recently been reviewed.[8,26] It is critical to demonstrate that monoclonal antibodies, when given intravenously, actually reach the tumor and show a degree of specificity for the tumor cells. While radioisotope-labeled antibodies can be used to image the tumor, sensitive immunologic techniques, such as immunoperoxidase and flow cytometry, provide direct evidence for localization of the antibody on the tumor-cell membrane. In this regard, patients with malignant melanoma represent a unique population because of the accessibility of their cutaneous tumor nodules for biopsy. The 9.2.27 antimelanoma monoclonal antibody was selected since it recognizes a melanoma-associated antigen (250,000-dalton chondroitin sulfate proteoglycan core glycoprotein) on most (90%) melanomas and is relatively selective for the tumor cells.[10] We have also recently conducted a Phase I study in chronic lymphocytic leukemia (CLL) and cutaneous T-cell lymphomas (CTCL) with T101 monoclonal antibody.[9,27] T101 binds to a 65,000-dalton antigen, found on circulating normal T-lymphocytes, malignant T-lymphocytes, and B-cell-derived CLL cells.

These initial studies were designed to focus primarily on antibody localization on tumor cells and the immunologic response to murine monoclonal antibody. Eight patients with malignant melanoma whose tumors reacted *in vitro* with the 9.2.27 monoclonal antibody were treated with escalating doses of antibody to demonstrate localization of antibody in skin nodules as determined by repeated biopsies performed after treatment. All patients were monitored for clinical response and toxicity, antibody pharmacokinetics, antigenic modulation, and antimurine antibody formation.

TABLE 10. Properties of Human Interleukin-2

Biochemical properties*		Biological properties
Sensitivity to treatments		Origin
Boiling	+	Mature helper T-cells
RNAase, DNAase	−	Macrophages required
Trypsin	+	Antigen/mitogen required
Freezing	−	Target cell
N-ethylmaleimide	−	Any activated T-cell
Dithiothreitol	−	Resting T-cell inactive
Hg C12	−	NK-cells
Stabilized by		Biological effect
Polyethylene glycol	+	Binds to specific receptor
Albumin	+	Promotes entry into S-phase
Glycerol	−	Stimulates proliferation
Molecular weight (SDS-PAGE)	12,000–13,000	Specifies specificity
Isoelectric point	6.8	Very little; active in other primates, cow, cats, rodents

Adapted from Russetti F. BRMP Lymphokine Retreat.
* Purified Interleukin-2 stabilized with polyethylene glycol was used.

In Vivo Localization of Monoclonal Antibody in Melanoma

Biopsies were performed from 1 to 4 days after infusion of 9.2.27. No antibody was detected in tumors after infusion of 1 mg of antibody; however, antibody was routinely detected by immunohistology in subcutaneous skin lesions after infusion of dosages of 50 mg or greater. Localization was related to quantity of antibody infused as the intensity and homogeneity of tumor staining increased with increasing dose. In addition, the diffusion of antibody out into the tumor nodule was clearly dose-dependent. *In vivo* localization of monoclonal antibody in subcutaneous tumors was detected in six of eight patients, and the flow cytometry data generally correlated with the immunoperoxidase data (Table 11). Flow cytometry also demonstrated that there was no antigenic modulation secondary to therapy. Tumor cells were not saturated with 200 mg of 9.2.27 antibody *in vivo*, but saturation was observed at 500 mg.

TABLE 11. *In Vitro* and *In Vivo* Reactivity of 9.2.27 Antibody With Melanoma Cells in Cutaneous Skin Lesions

Patient no.	Dose 9.2.27	Days posttreatment	Flow cytometry (%)		Immunoperoxidase	
			In vitro	*In vivo*	*In vitro*	*In vivo*
1	Pretreatment		80	ND	++	–
	1 mg	1	92	0	++	–
2	Pretreatment		83	ND	++	–
	1 mg	1	0	0	+	–
	50 mg	1	72	0	++	+
	200 mg	1	ND	ND	++	+
3	Pretreatment		97	ND	++	–
	10 mg	1	98	2	+	–
	100 mg	1	72	50	+	+
	200 mg	4	98	91	+	+
	Pretreatment		90	ND	++	–
4	200 mg	1	73	71	+	+
	Pretreatment		76	ND	++	–
	1 mg	1	91	0	++	–
5	200 mg	1	41	35	++	+

In Vivo Binding of T101 Antibody and Antigenic Modulation of the T65 Antigen

Thirteen patients with Stage IV chronic lymphocytic leukemia were treated. Three patients were removed from the study early because they experienced toxicity. *In vivo* binding of circulating mouse IgG (T101) to circulating leukemia cells was demonstrated in all patients treated. We compared *in vivo versus in vitro* staining in one representative patient from each treatment group (Fig. 5). In the lower curves, only fluorescein-labeled goat anti-mouse immunoglobulin was added to the cells (*in vivo* labeling). Excess T101 antibody was added to the same cell specimens (upper curve), followed by the fluorescein-labeled secondary antibody, to determine the percent labeling of cells *in vitro*. At 1 mg, a small proportion of circulating cells were stained *in vivo* (25%), whereas excess antibody stained 80% of the cells *in vitro*. Immediately after a 2-hour infusion of 10 mg of antibody, there was a maximum number of *in vivo* stained circulating cells, which equaled the number stained *in vitro*. This declined rapidly over the next few hours as T101 was lost from the cell-surface membrane or these cells were removed from circulation. Following a rapid infusion of 40 mg of T101, there was again maximum *in vivo* binding of T101 to circulating cells immediately after therapy. In contrast to the results obtained following 10-mg infusions over 2 hours, prolonged infusions of 50 and 100 mg of T101 monoclonal antibody (1–2 mg/hr) resulted in nearly 100% modulation of the T65 antigen secondary to this prolonged infusion, preventing binding of T101 antibody to the cell-surface membrane. This modulation was maintained throughout the infusion and prevented any significant reduction of the circulating cell count. Bone marrow and circulating cells recovered most of their T65 antigen expression by 24 hours after therapy.

Bone marrow samples were removed to determine *in vivo* localization of T101 antibody in leukemic bone marrow cells. Bone marrow cells were removed prior to infusion, as well as 2 and 24 hours following infusion of 10 mg of T101 antibody. Prior to therapy, 90% of the cells stained *in vitro* with T101 antibody. Eighty-

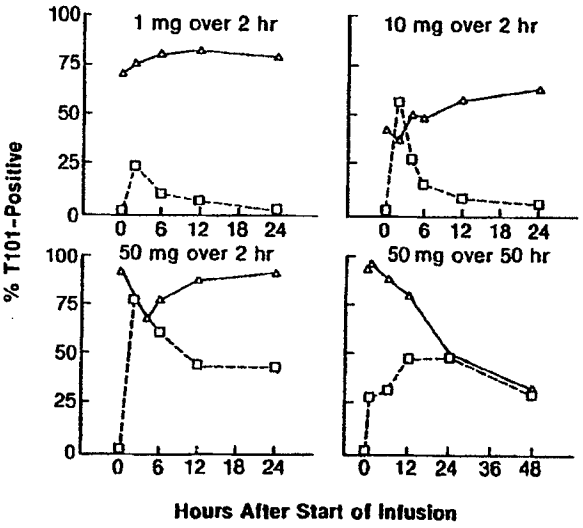

FIG. 5. *In vivo versus in vitro* binding of T101 antibody by circulating chronic lymphocytic leukemia cells. Results from one patient at each dose level are shown. The lower curve (□) represents *in vivo* labeling of circulating leukemia cells with T101 antibody. The upper curve (Δ) represents the same circulating leukemia cells treated *in vitro* with ex · · T101, and then labeled with fluorescein-tagged goat anti-murine antibody. The difference between these curves at each time point measured represents the number of cells that were not labeled *in vivo* with T101.

one percent of the leukemic bone marrow cells were labeled *in vivo* 2 hours after infusion of T101, with only 10% additional labeling when excess T101 was added *in vitro*. In addition, antigenic modulation was demonstrated in one patient's bone marrow tumor-cell population 2 hours after the 10 mg infusion of T101. Twenty-four hours after therapy, the bone marrow cells no longer had *in vivo* bound T101 on the cell-surface membrane and the cells had fully recovered expression of the T65 antigen.

Lymph nodes were removed from one patient 5 hours after a 2-hour infusion of 10 mg of T101 and from another patient at the completion of a 50-hour infusion of 50 mg of antibody. In neither case could we detect *in vivo* labeling of tumor cells or antigenc modulation of CLL cells in the lymph node, while in both cases >70% of the cells stained with T101 *in vitro*. These data strongly suggest that T101 did not reach the lymph node and did not label lymph node cells *in vivo*. This was not surprising, as we could not detect circulating free T101 in these patients' sera. In order to increase the amount of free T101 in the serum, one patient was treated with a 50-mg infusion of T101 over 50 hours to modulate his circulating and bone marrow leukemia cells. Immediately following this therapy, the patient was given 5, 10, 50, and 100 mg of T101 antibody, each by infusion over 1 hour. Therapy was stopped due to hypotension 15 minutes (25 mg) into the 100-mg dose of T101. At this point, 5.4 μg/ml of free T101 was measured in the patient's serum. A lymph node biopsy performed 1 hour after the completion of therapy demonstrated nearly 100% modulation of lymph node cells. These cells were cultured *in vitro* without T101, and after 5 days, 83% of the cells expressed the T65 antigen. these data strongly suggest that T101 reached these lymph node cells *in vivo* and induced antigenic modulation.

Pharmacokinetics and Antiglobulin Response

To assess the circulating levels of murine monoclonal antibody, sera of patients was assayed beginning at the end of infusion and 3 and 24 hours postinfusion. Peak values of 9.2.27 progressively increased with escalating doses. Interestingly, the peak after the 50-mg dose was much less than expected and correlated with a heightened IgG and IgM antiglobulin response. More importantly, the next escalated dose of 100 mg achieved high transient levels of 9.2.27, consistent with a saturation of the antiglobulin response. Circulating antibody levels were dose-related. Higher and more prolonged levels of 9.2.27 were achieved with higher doses of antibody. Further tests indicated that circulating 9.2.27 had not formed immune complexes with antiglobulin and could still bind to cultured melanoma cells.

The antiglobulin response did not impede localization of 9.2.27 in cutaneous nodules as there was an increment in the amount of bound antibody in nodules studied by biopsy following the 100- and 200-mg doses. Three of eight patients had measureable increases in antiglobulin responses (either of IgG or IgM) after administration of the 10- or 50-mg doses and a loss of demonstrable antiglobulin at the higher dose levels. In the other five patients there was no change in antiglobulin activity from baseline levels at any dose level of 9.2.27.

Serum specimens from patients receiving T101 were quantitated for human IgG, IgM, or IgA anti-T101 titers using an ELISA. While some of the CTCL patients demonstrated anti-T101 activity before therapy and all demonstrated detectable antiglobulin titers after therapy, none of the CLL patients had detectable antiglobulin before or after treatment. Further experiments revealed that the anti-T101 activity detected in both patient and normal control specimens was not specific for either the T101 antibody or mouse IgG_{2a} molecules in that similar degrees of reactivity were observed using IgG preparations of rabbit, rat, guinea pig, or goat origin. The lack of antiglobulin response in CLL may relate to the B-cell defect in that disease, but the reason for a lack of response in the five of eight patients with melanoma is less clear.

Measurable quantities of T101 were not detected in patients receiving less than 100-mg dose levels. The detection limit for the assay employed was in the range of 0.2 to 0.4 μg/ml. Both patients receiving 100-mg doses of T101 over 50-hour periods of infusion demonstrated measurable levels of T101. In both patients, detectable circulating levels of murine IgG were not obtained until 24 hours after starting the first infusion, reaching maximal concentrations of 1.0 and 3.5 μg/ml, respectively. After modulation (third infusion of 100 mg), circulating murine IgG was detected in two patients immediately before the initiation of the third dose and rose to levels of 4.2 and 5 μg/ml over the period of infusion.

Clinical Response

There were no measurable decreases in the size of the melanoma skin lesions, lymph nodes, or visceral metastases during treatment with 9.2.27, although some of the nodules softened in two patients.

In patients treated with T101, circulating leukemia cell counts routinely dropped to approximately 50% of prétreatment levels immediately following a 2-hour infusion. However, by 24 to 48 hours the cell count

generally recovered to the pretreatment level. Two of the patients treated with a 2-hour infusion of 10 mg maintained a reduction in their circulating leukemic cell count to the pretreatment levels throughout the course of therapy. Response in patients treated with prolonged infusions of antibody was quite different. These patients demonstrated a 20% to 50% drop immediately following the infusion of monoclonal antibody that reached its nadir approximately 12 to 24 hours into the infusion (25–50 mg of antibody administered). Cell counts gradually rose, and by the time the infusion was completed, cell counts had recovered to pretreatment levels in most patients. The circulating cell count correlated inversely with the degree of antigenic modulation of the T65 antigen. None of the patients demonstrated a reduction in the size of lymph nodes or organs or showed an improvement in platelets or erythrocyte counts. Some improvement in the skin lesions of CTCL patients was seen.[9]

Toxicity

All eight melanoma patients developed fevers of up to 39.4°C during 9.2.27 infusion. These fevers were well tolerated and did not require antipyretics. Mild nausea, anorexia, chills, and headache were seen in some of the patients. None of these patients developed immediate systemic allergic reactions such as generalized urticaria or anaphylaxis. Local urticaria was seen in two patients. There were no renal, hepatic, or hematopoietic abnormalities detected during or after the infusion of this antibody. One patient developed arthralgias, fevers to 38.8°C, headache, and a maculopapular erythematous skin rash on his trunk and extremities 10 days after his first dose of 9.2.27. This responded promptly to high-dose intravenous steroids and probably represents the first case of serum sickness associated with monoclonal antibody therapy.

Temperatures of up to 38.3°C were seen in most patients treated with T101 at all of the dose levels greater than 1 mg. Urticaria was seen in approximately 50% of the patients treated with T101 antibody at some point during the course of therapy. In all cases, this promptly responded to antihistamines and was generally prevented subsequently by pretreatment with antihistamines. Pulmonary toxicity was seen in all patients treated with 2-hour infusions of 50 mg of T101, including sudden shortness of breath with chest tightness without changes in chest x-ray or blood gas findings. This usually resolved completely without residual problems within 30 to 60 minutes after stopping therapy with or without treatment with antihistamines. When the infusions wre slowed to 1 to 2 mg/hour, this toxicity was no longer witnessed.

Conclusion

The use of biologicals and biological response modifiers represents the emerging fourth modality of cancer treatment.[1] In addition, these agents and this technology have far broader applications than cancer therapeutics. Given the large number of cloned biologicals and the virtually unlimited number of recombinant molecules that can be produced, along with the great variety of monoclonal antibodies that can be produced through hybridoma technology, it is apparent that the coming decade will produce new challenges for those involved in developing new anticancer therapies.[28] Over the last 30 years, only approximately 40 chemotherapeutic agents have cleared all of the preclinical evaluations and have been made available for standard clinical use. By contrast, it now appears that we will have a comparatively large number of new, potentially useful therapeutic biologicals available within the next decade.[29] This tremendous expansion of agents and approaches available for cancer therapeutics will increase our opportunities and amplify our problems with the preclinical evaluation of these agents and their transition to clinical use. New mechanisms for the development of these biologicals and the selection of those "most likely to succeed" need to be developed to conserve resources. Additionally, the methods used by pharmaceutical manufacturers and the regulations established by federal agencies will likely be inhibitory in the face of a large number of biologicals to be tested in humans. The development of novel approaches will be necessary if these biologicals are to be made available effectively and rapidly for clinical trials.[28]

The testing has begun. Immunomodulators, lymphokines/cytokines, and monoclonal antibodies are being evaluated extensively in preclinical models and in Phase I, II, and III clinical studies. Historically, the chemical, bacterial, and viral immunomodulators have not proven highly useful in clinical trials.[30] By contrast, it is already apparent that alpha interferon has evident antitumor effects even in certain patients with bulky disease.[31] This finding should give pause to those considering the immunologic dogma that biological response modifiers can only be active in minimal residual disease. It may be that, like chemotherapy and radiotherapy, biologicals and biological response modifiers may be more active with lesser tumor burdens; however, the early data indicate that activity and, hence, selection of compounds for further study can be assessed in patients with advanced disease.

A very clear demonstration that monoclonal antibody, when administered intravenously, can selectively locate on the tumor-cell membrane gives strong support to the

concept that these agents may be useful in specific targeting of cancer therapy. Already, investigations into the use of immunoconjugates indicate that monoclonal antibodies can be used to carry drugs, toxins, and radioisotopes to the cancer cell with a specificity heretofore unavailable in cancer therapeutics.[32-34]

These early findings with immunomodulators, lymphokines/cytokines, and monoclonal antibodies serve to indicate what a productive investigative era we are entering. Never before have so many approaches appeared to be so feasible, practical, and clinically useful. Many oncologists would agree that we have reached a plateau in cancer treatment using the three classic modalities of surgery, radiotherapy, and chemotherapy. The advent of biologicals and biological response modifiers as the fourth modality of cancer treatment will serve to move us from this plateau onto a more effective and more specific therapeutic approach to the treatment of cancer.[1]

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EXHIBIT 2

Targeted Drug Delivery

ARNOLD I. FREEMAN, MD,* AND ERIC MAYHEW, PHD†

Cancer chemotherapy drugs are neither specific, *i.e.*, they do not act exclusively on the metabolic pathways of cancer cells, nor are they targeted solely toward cancer cells. However, recent research has begun to address, in part, the latter issue. Improved delivery of chemotherapeutic agents to tumor tissue in man appears to be an achievable goal in the next decade. Improved drug delivery includes developing predictive models that allow for laboratory assessment of the best treatment for a patient's cancer without exposing the patient to an empirical trial or to the possible morbidity from exposure to a less useful drug, or to the loss of time in the fight against cancer because of ineffectual therapy. Monoclonal antibodies directed against tumor-associated antigens have the potential to achieve major advances in targeted drug delivery. Monoclonal antibodies may have direct antitumor effects, or they can be used as "homing devices" when attached to a payload and can guide diagnostic or therapeutic agents to the targeted tissues. Carrier systems of all types have become available; these include liposomes and polymeric compounds which can carry drugs, radionuclides, toxins, or other materials in a protected environment. These carriers can also be bound to monoclonal antibodies for possible targeted delivery. Pharmacological sanctuaries have been recognized as a problem in cancer treatment. The best known of these is the central nervous system (CNS). Techniques to temporarily disrupt the blood–brain barrier are now appearing. Mechanisms to administer therapy directly into the CNS are also being reassessed. Implantable pumps and reservoirs have been used to treat selected organs or for regional perfusions. Other treatments that are regional in scope include administration directly into a cavity or into a tumor. Computerized implantable devices should play a major role in cancer therapy in the future, in pain control as well as antibiotic and hormone administration. In recent years, mathematical models have been developed that can more accurately predict drug distribution and metabolism in various tissues of the body. Such models point the way to more logical designs of chemotherapeutic administration. The expanded use of autologous bone marrow transplantation, along with improving techniques of "purging" the marrow of tumor cells before reinfusion can be anticipated. Pro-drugs are substances that must be biotransformed *in vivo* to exert their pharmacologic effect. Certain pro-drugs that may be more effective or resistance-avoiding analogues of established chemotherapeutic drugs are currently under development and offer considerable promise. A new era of improved drug delivery is achievable and can lead to greater efficacy of treatment regimens and a higher cure rate while at the same time reducing toxicity. This discussion deals primarily with the currently emerging therapies of monoclonal antibodies, liposomes and intra-arterial infusions.

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IN RECENT DECADES, powerful drugs have been discovered that have achieved cures in some types of cancer and beneficial effects in others, albeit with undesirable toxicity to normal tissues. The influence of the dosage schedule on drug activity has been apparent from earliest recorded observations. The difference between success or failure in cancer therapy is often not just the use of a drug itself, but how the drug is used. This is not different from skill in the use of a surgeon's scalpel or a radiotherapist's beam. Unfortunately, due to the relatively nonspecific nature of the action of chemotherapeutic drugs, usually there is toxicity to normal tissue even under optimal conditions. Furthermore, to date, cancer chemotherapeutic drugs are neither specific, *i.e.*, they do not act exclusively on the metabolic pathways of cancer cells, nor are they targeted exclusively to cancer cells. However, recent research has begun to address, in part, the latter issue. We stand on the threshold of a new era where improved delivery of chemotherapeutic agents to tumor tissue in man appears to be an achievable goal that should result in improved survival and quality of life for the patients.

The area of targeted drug delivery includes developing predictive models that allow for laboratory assessment of the best treatment for individual cancer patients without exposing that patient to an empirical trial, or to possible

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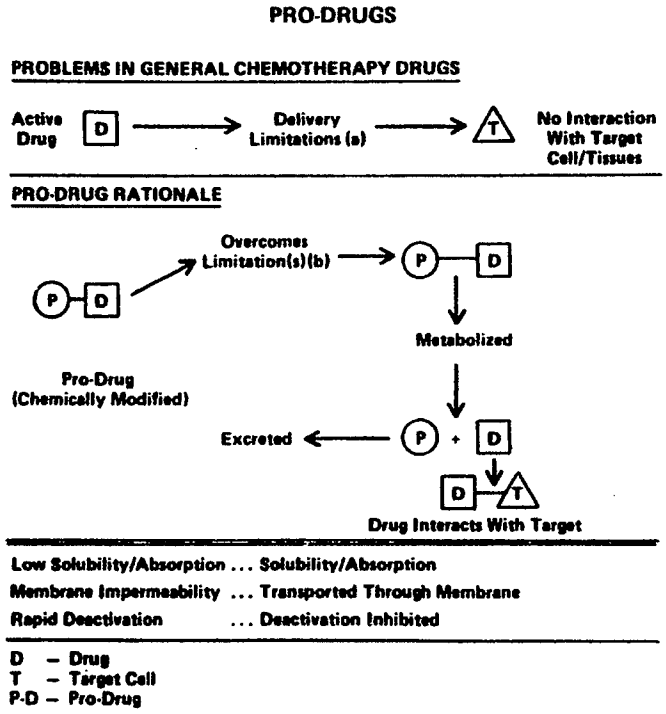

FIG. 1. Pro-drugs. Conceptual basis.

morbidity or loss of time in the fight against cancer due to exposure to a cancer-resistant drug. This article gives an overview of the current status of drug delivery.

Pharmacologic Sanctuaries

The problem of pharmacologic sanctuaries has long been recognized as a major concern, particularly where there are potential highly effective agents that cannot reach the tumor so no therapeutic effects are seen.

Improved delivery is needed for treatment of tumors that are located in these sanctuaries. The best known sanctuary is the central nervous system (CNS). Techniques to temporarily disrupt the blood–brain barrier (BBB) in order to enhance CNS uptake of systemically administered drugs are now appearing, and, mechanisms to administer therapy directly into the CNS are being reassessed. In addition, there have been recent developments in drug design that may be able to deliver an active drug into the CNS using the pro-drug principle[1] (Fig. 1).

TABLE 1. Local Delivery*

Regional therapy
Direct intra-arterial drug administration, *e.g.*, regional perfusion or regional infusion
Site selective administration such as intraventricular administration to the brain into a body cavity, *e.g.*, pleural, or directly into the tumor or topical application to involved skin
Drug carrier therapy
Organ selective depending on mode of administration, *e.g.*, colloidal carriers are preferentially taken up by the liver and spleen after IV administration and draining lymph node uptake after SC administration.
Cell selective where the carrier is taken up by specific target cells. Selectivity results from specific binding between carrier ligand and target cell receptor, *e.g.*, McAb and surface antigen.
The natural ability of cells of the RES specialized to take up colloidal particles results in selective delivery.

* Defined as administering the drug to an anatomical regiion or to an organ or part of an organ, wherein the tumor is located.
IV: intravenous; SC: subcutaneous; RES: reticulo-endothelial system.

Pro-Drugs/Soft Drugs

Pro-drugs are substances that must be biotransformed *in vivo* to exert their pharmacologic effects (Fig. 1). There are a number of already existing effective pro-drugs, but they were not initially developed as such. For example, cytosine arabinoside has to be phosphorylated *in vivo* in the target cell to the triphosphate that is the active moiety. The direct administration of triphosphate is not of clinical use because of its rapid *in vivo* dephosphorylation and poor intracellular transport. The alkylator, cyclophosphamide, is another example of a pro-drug because it has to be transformed in the liver to its active metabolite. Development of pro-drug analogues should give rise to useful agents, including agents that may overcome some types of drug resistance.

The balance between efficacy and toxicity is most important in cancer chemotherapy. Thus, in addition to pro-drugs, an important corollary is the concept of soft drug development.[2] Soft drugs are active therapeutic agents with enhanced inactivation *in vivo*, thereby reducing toxicity. A reduction in toxicity may be achieved by: (1) eliminating the formation of toxic metabolites, (2) eliminating drug interactions and, (3) simplifying pharmacokinetic problems resulting from multicomponent systems.[2] Successful soft drugs would decrease toxicity and lead to an improved quality of life for cancer patients.

Currently, one of the basic problems in cancer chemotherapy is the lack of knowledge of biochemical lesions specific to cancer cells for which agents could be specifically designed. Only after the detection of basic differences distinguishing cancer from noncancer cells can specific anti-tumor agents be formulated.

Targeted Delivery

Although the development of specific cancer drugs still lies in the future, significant gains should arise from targeted delivery of current agents, as well as, from future analogues specifically modified for altered delivery. There are at least two distinct types of delivery: (1) local delivery and (2) cell targeted delivery (see Table 1).

Local Therapy

Regional Therapy

Regional therapy has been used moderately frequently in the treatment of cancer. The basis for intra-arterial infusion is the increased drug concentration to the infused area and a simultaneous reduction of the drug going into the systemic circulation, which results in reduced systemic toxicity. Thus, tumor kill will be enhanced with a simultaneous reduction of toxicity. Another potential benefit from direct intra-arterial usage is that there may be a threshhold concentration that is needed to achieve tumor kill and reaching this peak level *in vivo* may be achievable only by direct intra-arterial administration. In a closed system, such as certain types of limb perfusion systems, there is very little return of the drug to the systemic circulation, but in most circumstances there is significant recirculation, so systemic toxicity may be reduced but not eliminated.

Regional Therapy: Pharmacokinetic Rationale

The basic assumption of increase drug concentration to a region or organ containing tumor was that higher drug concentrations would lead to greater cell kill, which in turn would lead to improved tumor response. Intra-arterial chemotherapy is the best-defined technique of achieving this goal. After the intra-arterial injection of a drug and after the drug has passed through the target site and become mixed with venous blood, any further exposure will be the same as if this drug had been administered *via* a peripheral vein. Any advantage, therefore, must be gleaned from the first pass.

In general, the therapeutic advantage of intra-arterial over intravenous (IV) administration depends on: (1) the fraction of cardiac output flowing through the infused artery (*i.e.*, the lower the arterial blood flow in this site, the greater the benefit for the intra-arterial route); and (2) the fraction of drug cleared from the systemic circulation during a single passage (*i.e.* the faster the total body clearance—metabolism and/or excretion—the greater the benefit for intra-arterial route.[3,4,5] However, if the chemotherapeutic agent is eliminated solely by the organ that has been infused, there may be no advantage to intra-arterial drug delivery as far as total amount of drug exposure to the tumor, but peak levels of the drug will be higher by the intra-arterial route (*e.g.*, the direct intra-hepatic artery infusion of doxorubicin (Adriamycin), where 90% of the drug is excreted by the liver). In this case although the total amount of doxorubicin exposure is the same by direct intra-hepatic arterial administration as with IV administration, the systemic toxicity may be substantially decreased following intra-arterial administration because a substantial amount of drug is removed from the circulation on the first pass through the liver. This allows for the use of higher doses of this drug. The intra-arterial infusion of doxorubicin into an extremity has the advantage of an increase in regional drug level.

5-fluorouracil (5-FU) is an agent that is cleared extremely quickly following IV injection, and by 3 hours, there is no detectable drug in the plasma.[6] The normal renal excretion of 5-FU is only 16% of the administered dose. The plasma, half-life of the drug is only 10 to 12 minutes. Studies have shown that, like doxorubicin, more than 90% of 5-FU is extracted by the liver.[7,8] Thus, direct intra-hepatic arterial infusions of 5-FU should be followed by lesser systemic toxicity than when the drug is given by IV.

Recently, there has been a flurry of activity in the use of intra-arterial therapy in brain tumors, in an attempt to deliver drug into the CNS. Recent reports of intra-arterial cisplatin, BCNU, VM-26 and other drugs appear to show a significant advantage[9] over IV administration.

Even direct intra-arterial chemotherapy *via* the pulmonary artery for pulmonary metastases has been attempted.[10] In this situation, doxorubicin *via* the pulmonary artery did produce significantly higher drug levels in infused canine lung than did systemic administration.

Intra-arterial infusion for some drugs is not advantageous. For example, when bleomycin is administered either through the intra-arterial or IV route, approximately 40% to 70% of the administered dose is excreted, unchanged, in the urine within 24 hours.[11,12] The time of total body clearance from the plasma was estimated to be approximately 87.5 minutes.[11] On a theoretical basis therefore, this drug should prove to have minimal or no benefit when given by regional intra-arterial routes.

In recent years, intra-arterial infusions to the extremities have been used in conjunction with a tourniquet.[13] Here, the external tourniquet is applied to the extremity for 10 minutes. This results in much higher drug levels in the infused extremity. The use of this technique may make it possible for certain agents whose therapeutic margin is only increased minimally by intra-arterial infusion, to have further significant benefit with restriction of returning venous blood flow.

When drugs are injected into the peritoneal cavity, it was shown that the transport across the peritoneal membrane depended primarily upon the molecular weight of these drugs.[14] Collins and Dedrick noted the similarity in quantitative models for intraperitoneal, intrathecal, and intra-arterially administered anticancer drugs.[15] However, unlike in the intra-arterial route, only cells within body cavities in direct contact with drug will be exposed to high concentrations of the chemotherapeutic agent in the other two approaches. Tumor cells located further away will lose this therapeutic benefit.[16,17]

Nonetheless, the critical and largely unanswered ques-

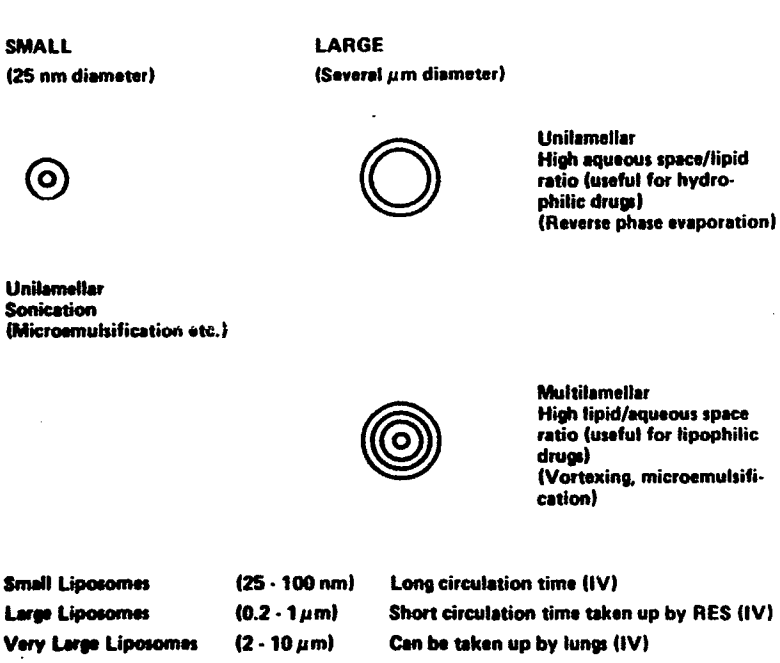

FIG. 2. Liposomes. Size, structure, and common type of manufacturer.

Small Liposomes	(25 - 100 nm)	Long circulation time (IV)
Large Liposomes	(0.2 - 1 μm)	Short circulation time taken up by RES (IV)
Very Large Liposomes	(2 - 10 μm)	Can be taken up by lungs (IV)

tion remains whether higher drug concentrations will result in an increased tumor cell kill. In acute lymphocytic leukemia, higher concentrations of methotrexate (approximately 10^{-4}) when administered intrathecally eliminate visible leukemic cells from the cerebral spinal fluid (CSF) whereas leukemic cells appear uneffected in the blood and bone marrow by lesser concentrations of methotrexate (approximately 10^{-7}).[18-20] This observation in acute leukemia indicates the benefit of higher drug concentrations of effective chemotherapeutic agents in responsive tumors.

Drug Carrier Therapy

There are a number of drug carriers including proteins, glycoproteins, nucleic acids, starch particles, various synthetic polymers, and liposomes. We shall discuss liposomes in greater detail because they have some unique characteristics and are a model system for a number of other carriers.[21]

Liposomes

Liposomes (lipid vesicles) were first described by Bangham *et al.*[22] They can be made from naturally occurring as well as synthetic phospholipids, are biodegradable, and are relatively nontoxic. They can be manufactured relatively easily, in a range of sizes from 0.03 to 10 microns, and require no chemical bond formation during liposome drug preparation (Fig. 2). Small size has advantages for more prolonged circulation time and greater likelihood of reaching the interstitial space in targeted areas, but has the disadvantage of carrying less entrapped (water soluble) drug. Large multilamellar liposomes tend to aggregate in the lungs, and the very large liposomes (approaching 3–10 microns) are substantially more toxic in mice when significant amounts are injected IV, perhaps due to lung embolism.[23] Methods for preparation of large unilamellar vesicles have also been developed.[24,25] At the cellular level, liposomes are initially absorbed at the cell surface and are subsequently taken up by endocytosis and possibly by other mechanisms such as fusion between the liposomal and cellular membranes. The size, chemical composition and surface charge of liposomes can alter their characteristics. Liposomes can be produced possessing a surface charge running from positive to neutral to negative, which can alter their pattern of distribution in the body. They can be made with phospholipids that vary in the fatty acid chain configuration so they can be either solid or fluid at defined temperatures. Generally, liposomes that are solid and those containing higher ratios of cholesterol are more stable than other types of liposome.[25,26] Also, the glycolipid composition of liposomes can alter their affinity for various tissues.[27-29]

In general, when liposomes are to be used as carriers, it is best to have a well-defined and fairly consistent size; this is readily achieved by passing the liposomes through nucleopore polycarbonate membranes.[30] More recently, a microemulsification method for rapid preparation of liposomes of defined size has been described.[31]

After IV administration, liposomes are first filtered through the lung and after passage, they are distributed to the body where they are cleared mainly by the liver

and spleen, although most organs take up liposomes and their content to a limited extent. In general, large liposomes are cleared more quickly than small ones, and positive and neutral liposomes are cleared more slowly than negative liposomes.[32,33] Furthermore, it has been shown that, depending on their construction, liposomes can be made to release entrapped drugs at increased temperatures.[34-38] This increases the potential for regional therapy because parts of the body can be made hyperthermic with various techniques. Based on observations that the pH may be reduced in tumors, liposomes have been made that will release their contents much more efficiently at pH 5 to 6 than at pH 7.2, with the intent of specific release of drugs, only in tumors.[39,40]

In man, empty liposomes have been injected intravenously in cancer patients.[41] It was found that over 40% of the labeled liposomes sequestered in the liver followed by spleen (25%), and kidney (14%), and then bone marrow. The cumulative urinary excretion of radioactivity was 13% over 24 hours. In this study, liposome administration was safe and devoid of significant side effects.[41]

One of the current problems effecting the use of the liposomes, is that solid tumors do not have a particular affinity to take up liposomes, and, in fact, the uptake may be less than that of surrounding normal tissue.[42,43] *In vivo,* tissues that absorb liposomes are rich in endocytotic cells,[44] and much of the uptake is by these cells. However, if the reticulo-endothelial system (RES) is depressed by prior saturation with drugs or silica, there is only moderate reduction of liposome uptake by tissues of the RES. This suggests that at least part of the uptake of liposomes by this tissue is due to factors other than endocytosis. In addition, insertion of specific liquids into the liposomal surface may help direct liposomes away from RES cells. Thus, it can be seen that even without attempting to specifically target liposomes with bound monoclonal antibodies, for example, tumors of the liver, spleen, lung, kidneys, and bone marrow may be susceptible to liposome drugs because liposomes normally accumulate there in significant quantities. Further, large liposomes may accumulate preferentially in the lung and be useful for lung tumor treatment, although the potential toxicity problem mentioned earlier would have to be overcome.

It has been emphasized that liposomes may be more than an inert vehicle, in that liposomes can alter drug distribution based on their size, chemical composition, particular nature, and surface charge.[45] When liposomes become associated with the cell plasma membrane, they can modify the cell membrane phospholipid composition. Similar effects may be seen during phagocytosis. These changes may alter enzymes and carriers in the cell membranes resulting in the liposomal lipids becoming an active participant in the therapeutic maneuver.

Because drugs packaged in liposomes are protected from environmental factors, they are likely to act to a variable extent as a depot or time-release system.[46,47] A number of cancer chemotherapeutic and antimicrobiologic agents have been trapped in liposomes and tested for activity in animal models.[48] The most extensively studied agents have been cytosine arabinoside (Ara-C), methotrexate, doxorubicin, dactinomycin, and amphotericin B. In the case of Ara-C, a single dose of Ara-C entrapped in liposomes significantly prolonged survival time in mice L-1210 tumor, and was at least as efficacious as the effects of free Ara-C administered as a prolonged IV infusion at optimal dosing.[49] It appears that much of the benefit here was to be related to the depot action of Ara-C entrapped in liposomes.

Results using liposome-entrapped doxorubicin in animals are also encouraging. A number of laboratories have shown that systemic toxicity is reduced, probably related to reduced cardiotoxicity, and that the therapeutic effects are maintained or enhanced against a number of different tumors, so that the therapeutic index is significantly increased.[50-54] Also, liposome-entrapped host-defense modulator muramyl dipeptide (MDP) and its lipophillic derivatives have been reported to have strong antimetastatic activity[55,56] in experimental systems.

Recently, it has been reported that amphotericin B encapsulated in liposomes was substantially less toxic than free amphotericin, and there was a higher cure rate in mice infected with fungal diseases compared with the effects of free amphotericin.[57,58] It is apparent that this is an area of major interest, and potentially great benefits could accrue from this therapy for cancer patients where candidiasis is a serious problem.

Routes of administration other than the IV have been investigated. Liposomes have been administered intraperitoneally (IP) where they are subsequently drained by the lymphatic circulation as well as by the capillary circulation and are taken up into mediastinal lymph nodes. Thus, there appears to be potential benefit for treatment of lymph node metastasis using the IP administration route in, for example, tumors that metastasize to regional nodes, such as in gonadal tumors. Liposomes have also been injected subcutaneously or *via* foot pad lymphatics. When injected by foot pad, accumulation of liposomes has been noted at the draining lymph node.[59,60] Liposomes have also been injected intra-arterially[61,62] and intra-cerebrally,[63] although extensive therapeutic studies have not been reported.

Polymers and Microspheres

Controlled-release polymers such as ethylene-vinyl acetate copolymer can release bioactive macromolecules for periods of several months or longer.[64] These systems have been suitable for the delivery of agents only in microgram or nanogram amounts.[65]

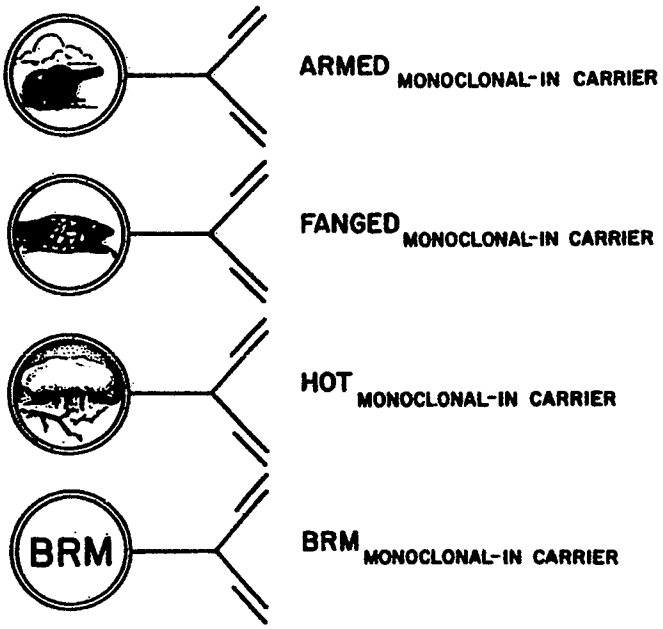

FIG. 3. Monoclonals. Use as a "homing device". In this case, monoclonals are attached to liposomes and then filled with chemotherapy (armed), toxin (fanged), radionuclide (hot) or biological response modifier (BRM).

Ethylene-vinyl acetate copolymer is impermeable to compounds with molecular weights greater than a few hundred daltons.[64] During the casting process, pores are formed in the macromolecular-polymer suspension due to the presence of particulate macromolecule. If small amounts of macromolecules are present, few pores are formed. On the other hand, if large amounts of macromolecules are present, pores are numerous. The pores interconnect to form tortuous channels that run through the matrix to the surface through which macromolecules can be released.[65]

Thus, polymers are in general valuable for release of compounds of small molecular weight. They are frequently implanted subcutaneously, and therefore cannot be used for a number of chemotherapeutic agents that must be given directly into the blood due to severe local toxicity when not administered directly into the blood. However, implants can be removed, such as after development of toxicity, whereas injected carriers, such as liposomes, cannot. These polymers may have a role in the future therapy including endocrine manipulation or pain control.

Recently, starch microspheres have been evaluated for intra-arterial drug delivery of chemotherapy.[66] These microspheres are approximately 15 to 100 microns in diameter. They may be combined with drug or radionuclide, and when infused intra-arterially, they form a starch gel that occludes the artery. This increases the concentration of drug in the target area because the drug is bound by the starch gel occluding the artery and is released as the starch is digested by circulating enzymes amylase. Recently, investigators from Japan reported objective tumor response after hemoembolization of kidney, bone, and liver cancer with mitomycin C microspheres. They observed embolization and infarctions of small arteries from ethyl cellulose capsules which resulted in the slow release of drug into the surrounding tumor tissue.[67,68]

Cell-Selective Delivery

The major goal for drug delivery is to selectively transport the agent to the specific intracellular site in all target cells without any delivery to nontarget cells. This is easier said than done. Several different approaches are possible, but the one with the highest likelihood of success is the use of monoclonal antibodies as the targeting determinants.

At the turn of the century, Paul Ehrlich speculated on the potential use of antibodies as carrier of pharmacologic drugs.[69] In the past, one of the major limitations to the use of antibodies was the inability to prepare sufficient quantities with sufficient specificity. Following the epic advance of Kohler and Millstein, who described the production of monoclonal antibodies (McAb) using the hybridoma technique,[70] new possibilities have opened for their evaluation in the diagnosis and therapy of cancer. The possible uses of McAb in cancer therapy can be divided, into a number of subclassifications (Fig. 3) when given alone *in vivo* as in serotherapy, or as a homing device when attached to a carrier that in turn is filled with: (1) a chemotherapeutic agent (armed), (2) a toxin (fanged), (3) a radionuclide (hot), or (4) a biological response modifier (BRM). In fact, McAb can be linked directly to some of these agents without a carrier.

It is not the purpose of this article to discuss the methodology of McAb production. In brief, animals, generally rodents, either mice or rats, are immunized with the antigen under consideration. Spleen cells from the immunized animals are fused with myeloma cells using polyethlene glycol as a fusion promoter.[71] There is selection of some of the fused myeloma spleen cells for hybrids capable of synthesis and secretion of antibody that binds the antigen with prerequisite affinity. The most frequently employed myelomas are resistant mutants that lack the enzyme hypoxanthine guanine ribosyl-transferase or thymidine kinase. The antibody-producing hybrid has chromosomal material from both cell lines fused into one nucleus. It should be emphasized that McAbs, although exquisitely specific when compared to the antibodies produced by older technologies, are not mono-specific. Consequently, investigators need to evaluate the McAbs against an appropriate panel of cells in order to accurately define the specificity of each McAb. Indeed, currently,

there is no one McAb that has been developed that is absolutely specific to a single tumor, nor to all tumor cells in a given population.

Clinical use of McAbs prepared using mouse systems represent exposure of patients to xenogeneic proteins. As a result, approximately half of the patients who have been treated with repeated administration of mouse McAbs have themselves produced a significant antibody response against mouse McAbs.[72] Furthermore, these human anti-mouse antibodies have rendered ineffectual the treatment with mouse McAb.[72] It may be possible to avoid this phenomenon by avoiding repetitive courses of mouse McAb, *i.e.,* by utilizing intensive therapy given over a brief time period, with these agents employed more as a "homing device". It may also be feasible to eliminate the antigenicity of the mouse McAb by "arming" it with a chemotherapeutic drug. Such "armed McAb" could eliminate any cells to which the antibodies become attached either tumor or normal immune cell. A number of investigators have suggested that the use of human monoclonals in place of mouse McAb would mute the antibody response because the "constant region" of the McAb would be recognized as self rather than a foreign protein. Consequently, there is significant interest in the development of human monoclonal antibody lines. However, to date, the human lines have yielded hybrids that result in reduced antibody production when compared to the rodent system.

Clinical Experience with Monoclonal Antibodies

Monoclonals as serotherapy *in vivo* has been tested in patients with far-advanced leukemia and lymphoma. Acute lymphocytic leukemia (ALL) has been treated with anti-CALL-A,[73] cutaneous T-cell lymphoma, T-cell ALL, and B-cell chronic lymphocytic leukemia have been treated with anti-Leu-1,[72-75] and nodular poorly differentiated lymphomas have been treated with anti-idiotype.[76] In general, toxicity has been modest. However, the use of mouse McAb has resulted in anti-mouse antibody that appears to block the therapeutic benefits of the monoclonals.[72] There have been reductions of the peripheral blast counts in the leukemic patients but, remissions are very rare. However, one patient with a follicular lymphoma was treated with a monoclonal anti-idiotype antibody and experienced a complete remission that lasted for over one year.[76]

The mechanism of *in vivo* tumor destruction by McAbs is unclear. It is apparently not due to lysis mediated by complement. It has been theorized that stimulation of natural killer (NK) cells or macrophages are responsible for the elimination of the tumor cells.

Problems have arisen after serotherapy in man. These include: (1) antigenic modulation, a reversible loss of antigenetic expression on the surface of tumor cells;[77] (2) immunoselection, *i.e.,* patients whose cells do not express the antigenic site are not attacked and continue to grow; (3) a shortage of effector cells, *i.e.,* null cells, natural killer cells or macrophages, and (4) the development of blocking factors.

Autologous Transplants

One of the major difficulties when using allogeneic bone marrow transplantation (BMT) for the treatment of cancer is the development of graft *versus* host response. Further, only approximately 40% of families have matched donors that are suitable for allogeneic BMT. There has been an attempt, however, to expand the use of autologous BMT. In order to do this, the bone marrow must be purged of tumor cells. One such purging method involves the use of McAb; that is, the bone marrow contaminated by tumor cells is removed from the patient and is treated *in vitro* with McAb. A number of the above problems with the use of *in vivo* serotherapy with McAb can be avoided when the *in vitro* technique is used. A potential limitation of this approach is that not all leukemic cells or other cancer cells are likely to express a single antigen, and it may be necessary to expose the bone marrow of these patients to a panel of antibodies *in vitro* to obtain their total elimination. In addition, it may be beneficial to "fang" the McAb with toxins. Other approaches to purging contaminated bone marrow in autologous transplants have avoided the use of McAb and, instead, have utilized chemotherapeutic drugs such as cyclophosphamide analogues, or lectins.

Fanged Monoclonals

McAb bound to toxins obviate the need for an effector cell such as the NK cell or the macrophage. The commonly employed toxins are ricin, abrin, and a diptheria toxin.[78,79] The former two are plant toxins and the latter a bacterial toxin. These toxins are comprised of two polypeptide chains. The A-chain is a biological active component that inhibits protein synthesis. The B-chain is a lectin that binds to the glycoproteins on the cell surface allowing the A-chain to enter the cell; the precise mechanisms of this are not clearly understood. In general, in the preparation of a fanged McAb, the A-chain is cleaved from the B-chain, and the A-chain is then covalently linked to the desired McAb. This reduces the danger of nonspecific binding of the B-chain to normal cells with their subsequent destruction. At the present time, because of possible danger from these potent toxins, fanged McAb have been utilized *in vitro* rather than *in vivo.* They do show considerable promise in autologous BMT[80] where the *in vivo* risk is avoided. Methods may be devised to reduce or eliminate possible nonspecific toxicity.

PHOTOTOXIC EFFECT

↓

PHOTOSENSITIZER + LIGHT
(porphyrin) (visible)

↓

ENERGY ABSORPTION

↓

EXCITATION

↓

ENERGY TRANSFER

↓

SINGLET OXYGEN PRODUCTION

↓

IRREVERSIBLE OXIDATION OF
BIOLOGICAL MATERIAL

FIG. 4. Principle of photoradiation therapy.

Monoclonals as Homing Devices

McAb may also be bound to the surfaces of macromolecular carriers, such as liposomes. The liposomes can then be loaded with radionuclide, toxin, or a chemotherapeutic agent. The possible advantages of this are substantial. The liposomes provide a protected environment for these agents, preventing degradation during their passage in the blood stream and, simultaneously, providing a protected environment for the host. Combinations of McAb can be coupled with the same liposome, and may result in a much greater binding affinity to the targeted tumor. An observed advantage[81] was that McAb bound to liposomes did not produce an anti-idiotype response in mice such as that observed when monoclonals by themselves were injected, although this work awaits confirmation. (The idiotype is the variable region on the immunoglobulin chain—both light and heavy chains.)

The potential usefulness of specific cell targeted agents in treatment of metastatic cancer cannot be over emphasized. The use of agents that will detect and destroy disseminated tumor cells in different regions of the body should be able to improve the cure rate. Site-directed treatment for some types of cancers known to spread *via* a cascade route could also be useful.

Photoradiation Therapy

Photoradiation therapy (PRT) has the ability to select tumor cells for destruction. Raab, in 1900, first reported a lethal effect on paramecia exposed simultaneously to light and an acridine dye.[82] Since then there have been reports of a photodynamic effect using a wide range of photosensitizers.[83] For this effect, oxygen generally is required in addition to a photosensitizer and light. It is theorized that there is an energy transfer from the excited triplet state of the sensitizer to oxygen, producing singlet oxygen that irreversibly oxidises subcellular elements (Fig. 4). A radius of up to 1 to 1.5 cm of tumor from the light source can be destroyed whether the light is administered superficially or interstitially. An example of lung cancer treated in this fashion is shown in Figure 5. Thus multiple interstitial treatment with multiple optical fibers (500 mw

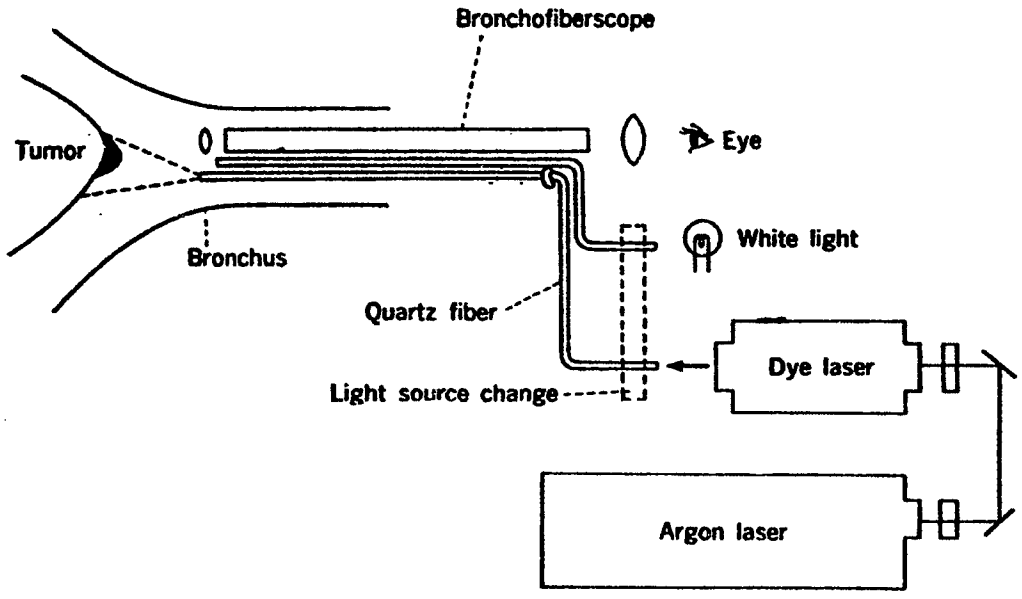

FIG. 5. Therapeutic system for the treatment of lung cancer with Hpd and light. The endoscope is inserted to the target using conventional white light illumination, then the beam from the argon dye laser system is photoradiated with or without the white light illumination. The tip of the fiberscope is usually maintained 1–2 cm from the target.

per cm length of diffuser) are needed to attempt to destroy tumors of large volume, not located on a body surface.

Ideally, for clinical use, a photosensitizer should be nontoxic, be selectively taken up and/or retained in cancer tissue, be activated by light able to penetrate to the lesion from the source, and be photochemically efficient. To date, the hematoporphyrin derivative (Hpd) and its purified active component (DHE) meet these criteria best. In experimental animals, PRT therapy using Hpd or DME is effective against a number of different types of tumors.[84,85]

After injection, the distribution of Hpd in various organs, as defined by fluorescence, is not consistent with the distribution as defined by radioactivity tagged porphyrins; for example, although little Hpd fluorescence was observed in liver, kidney, and spleen after administration of Hpd, these organs took up more ^{3}H- or ^{14}C-Hpd than any other tissues.[84] In at least some spontaneous tumors in animals and man, the Hpd uptake is considerably higher than that measured in induced rodent tumors.

Investigators from Roswell Park Memorial Institute were among the first to describe the efficacy of PRT in the treatment of superficial cutaneous and subcutaneous cancers.[85,86] PRT has proven effective in early-stage lung cancer, but in more advanced cases the results are thus far inconclusive. PRT has also been employed in early-stage esophageal cancer with encouraging results. PRT has demonstrated benefits in treatment of tumors of the skin, bronchus, trachea, esophagus, and bladder.[84,87-95] A major advantage is that PRT can be applied effectively and safely after other modalities no longer are effective, including its use in maximally radiated sites. Although it can theoretically be used to eradicate relatively large tumors, at the current level of sophistication, PRT appears especially advantageous in localized disease of relative small bulk. Because Hpd is retained for long periods of time in the skin, patients receiving Hpd need to keep out of sunlight to avoid severe sunburn.

In summary, it appears we are on the verge of developing new cancer chemotherapies that increase the ability to target drugs to the tumor under attack. This targeted drug delivery would simultaneously increase tumor kill and decrease toxicity. A variety of techniques for achieving targeted drug delivery are already available, and could make use of agents currently being used. Time will determine which of these techniques remain viable. Nonetheless, it does appear that significant improvement in the treatment of patients with cancer will be achieved *via* targeted drug delivery.

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EXHIBIT 3

United States Patent [19]

Ferris et al.

[11] **Patent Number: 4,771,128**

[45] **Date of Patent: Sep. 13, 1988**

[54] **METHOD OF PURIFYING TOXIN CONJUGATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY**

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[58] **Field of Search** 530/412, 387, 417, 391, 530/413, 388; 424/85

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[57] **ABSTRACT**

A method of isolating and purifying toxin conjugates using hydrophobic interaction chromatography. Crude conjugate mixtures are sized to remove unconjugated toxin, and loaded onto a column filled with a suitable hydrophobic gel. Elution is effected with salt solutions of decreasing ionic strength, which salt solutions optionally include increasing amounts of an organic solvent. Toxin conjugate substantially free of unconjugated Ig and unconjugated toxin is provided.

17 Claims, No Drawings

METHOD OF PURIFYING TOXIN CONJUGATES USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

FIELD OF THE INVENTION

This invention relates generally to chromatographic purification of toxin conjugates, and more particularly relates to a novel method of isolating and purifying immunoconjugates having hydrophobic interaction chromatography.

DESCRIPTION OF THE PRIOR ART

Conjugation of antibodies to toxic drugs and proteins in order to selectively kill tumor cells is an area of research that has recently become of some interest. To a large extent, this is due to the relatively recently developed ability to produce monoclonal antibodies using hybridoma technology, which antibodies are highly specific and can recognize tumor-associated antigens. "Immunoconjugates" may be prepared by covalently linking these antibodies to any of a number of cytotoxic agents. By conjugation, the affinity of the toxins for particular types of tumor cells is increased and the toxins can then exert their effects selectively, by virtue of the specific antibody carriers, against those cells.

Attention has specifically be focused on the highly toxic ribosome-inactivating proteins such as ricin (*Ricin communis*, extracted from castor beans). Preparation of immunoconjugates using these proteins is described, inter alia, in Miyazaki, H., *Gann* 71: 766–774 (1980) and Lambert, J., *J. Bio. Chem.* 260 (22): 12035–12041 (1985)). Ricin consists of two sub-units, termed "A-" and "B-" chains, which are linked by a single disulfide bond. While the A-chain has been shown to be solely responsible for cell death by catalytic inactivation of ribosomes, the B-chain has been demonstrated to provide a binding function, i.e., that chain is able to bind to cell-surface carbohydrates and thus promote the uptake of the A-chain into cells. In order to prepare a suitable immunoconjugate from ricin, then, it is necessary to bind the ricin A-chain to a specific cell-surface binding carrier such as an immunoglobulin (Ig).

Such Ib/ricin A-chain immunoconjugates are known (see, e.g., Miyazaki, supra). Isolation and purification of these immunoconjugates has, however, proved difficult. In prior art methods, while some amount of unreacted A-chain has been removed from the conjugation mixture, unreacted Ig remains in solution, contaminating the immunoconjugate preparation. Other investigators have succeeded in preparing a purified Ig/ricin immunoconjugate; however, that purification process necessitates a multi-step procedure including an ion exchange step (see Lambert, supra). The latter system further requires modification of pH and ionic strength for each conjugate. The present invention is directed to a more versatile and straightforward method of purifying immunoconjugates, which method removes substantially all unreacted antibody and protein from the conjugation mixture. The method uses hydrophobic interaction chromatography as the isolation and purification technique.

Hydrophobic interaction chromatography is a separation technique in which substances are separated on the basis of differing strengths of hydrophobic interaction with an uncharged bed material containing hydrophobic groups. Typically, the column is first equilibrated under conditions favorable to hydrophobic binding, e.g., high ionic strength. As the sample is eluted, a descending salt gradient is applied.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a method of isolating and purifying toxin conjugates using hydrophobic interaction chromatography.

It is another object of the present invention to provide a straightforward and versatile method of removing unreacted Ig from a crude conjugate mixture.

It is still another object of the invention to provide a method of isolating and purifying immunoconjugates purified by hydrophobic interaction chromatography, which immunoconjugates are substantially free of unreacted Ig and have high specific activities.

Additional objects, advantages and novel features of the invention will be set forth in part in the description which follows, andin part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

In one aspect of the present invention, immunoconjugates are prepared using known techniques. In preparing these immunoconjugates, monoclonal antibodies of the IgG class produced by hybridoma cells are linked by a disulfide bridge to a ribosome-inactivating or otherwise cytotoxic protein. The conjugate mixture, which contains unconjugated Ig and protein, including cytotoxic protein, as well as the immunoconjugate, is then purified by at least one chromatographic step. This process involves first removing unconjugated protein via sizing chromatography, followed by hydrophobic gel chromatography. In this latter step, the conjugate mixture is loaded onto a column packed with a gel containing hydrophobic groups, which column is capable of selectively retaining materials of different hydrophobic strengths. The individual components of the conjugate mixture are removed by eluting with salt solutions of decreasing ionic strength and, optionally, increasing amounts of a suitable organic solvent. Immunoconjugate substantially free of both unreacted Ig and unreacted cytotoxic protein is provided.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a novel method of preparing toxin conjugates substantially free of unconjugated protein. This method, in contrast to known methods, is particularly useful in removing unconjugated Ig and unconjugated protein from crude immunoconjugate mixtures.

Immunoconjugates which may be purified by the novel method include ribosome-inactivating proteins such as ricin A linked by a disulfide bridge to an Ig. Other protein toxins may also be linked by similar disulfide bridges, or thioether linkages in some instances, to form the immunoconjugate. Such other toxins include bacterial toxins, e.g., Pseudomonas exotoxin A and Diphtheria toxin, and plant toxins, for example momordin and saponarin. In addition, certain cytokines such as tumor necrosis factor (TNF) are cytotoxic. It has been found, however, that immunoconjugates of PAP, a toxin obtained form *Phytolacca americana*, cannot be purified by the method of the present invention.

Immunoconjugates, for purposes of the invention, may be defined as an antibody or antibody fragments such as Fab and $F(ab')_2$ that selectively bind to an epi-

tope, covalently bound to a protein toxin. More generally, toxin conjugates may be prepared by the method of the invention. A "toxin conjugate", as used herein, means a protein toxin covalently bound to a selective binding molecule. Such selective binding molecules may include, in addition to antibodies and the selective binding fragments thereof mentioned above, hormones, cytokines such as TNF, lymphokines such as interleukin 1 or 2, and cell growth factors such as transferrin, epidermal growth factor and bombesin. Such selective binding molecules bind to receptors found on the target cells to which these molecules bind. Immunoconjugates also selectively bind to cells; however, such binding is based generally upon affinity and avidity for a particular epitope associated with the target cell to which the immunoglobulin portion of the immunoconjugate binds.

While suitable method of preparing such immunoconjugates are known in the art (see, e.g., Miyazaki et al., supra, Lambert et al., supra, and U.S. Pat. No. 4,340,535 to Voisin et al.), a brief summary of the procedure used by applicants follows.

Monoclonal antibodies (designated in the Examples below as MAB260F9) of the IgG class were provided in a phosphate EDTA (P_iEDTA) solution containing about 0.10M Na_2PO_4 and 1 mM (minimum) EDTA. In order to prepare the antibodies for coupling to the free thiol on the ricin A chain, the Ig was derivatized with DTNB (dithionitrobenzoic acid) and iminothiolane (IT), at about 0° C. for a reaction time of about 24 hours. The Ig-TNBIT complex was then desalted using a Trisacryl GF-05 column (LKB, Bromma, Sweden) buffered to a pH of about 8.0 with P_iEDTA.

Soluble recombinant ricin A (srRTA) was provided by the method described in co-pending application Ser. No. 837,583 for "Recombinant Ricin Toxin Fragments." filed Mar. 7, 1986 and of common assignment herewith. The disclosure of that application is hereby incorporated by reference in its entirety.

The srRTA, at an initial concentration of about 10 mg/ml in P_iEDTA containing 0.1% β-mercaptoethanol (BME), was clarified by centrifugation (~1000 rpm) and desalted on a Trisacryl GF-05 column as above. A free thiol assay was run using DTNB and uv spectroscopy to assay released TND (peak at 412 nm).

The immunoconjugates were then prepared by adding about 10–30 vol. % of glycerol to the srRTA, followed by addition of the Ig-TNB-IT complex. The crude conjugate mixture was allowed to sit at room temperature for about 2 hours, at which time the conjugation process was presumed to be complete.

Purification of the crude conjugate mixture and removal of unconjugated Ig is carried out as follows:

According to the purification method of the present invention, the crude conjugate mixture as prepared above is first loaded onto a sizing column to remove unreacted srRTA and any high molecular weight aggregates. A suitable column for this step is Sephacryl S-300 (Pharmacia, Inc., Piscataway, N.J.), preferably equilibrated prior to use with a phosphate buffer (pH between about 6 and 7). The eluted conjugate mixture, in P_iEDTA, is at this point loaded onto a column preequilibrated in the same solution as the conjugate mixture outlined above, further containing 1 M NaCl, and packed with a relatively strongly hydrophobic gel such as Phenyl Sepharose CL-4B ® (manufactured by Pharmacia) or TSK Phenyl-5PW (Toyo Soda Kogyo K.K.).

With a Phenyl Sepharose column, the buffer used in both the sizing step and the subsequent chromatographic separation step preferably contains sodium chloride. With TSK Phenyl-5PW, ammonium solfate is the preferred alternative. Initial concentration of the salt is preferably about 1 M, the concentration used gradually decreasing with each column volume eluting the conjugate from the hydrophobic gel.

Immunoconjugate and unconjugated Ig are then separated and removed from the column as follows. Between about 4 and 10 column volumes of salt solutions (as above) successively decreasing in salt concentration are used to elute the various species. Optionally, increasing concentrations of an organic solvent such as glycerol, ethanol or propylene glycol may be added to the eluant solution to obtain the conjugate mixture in a more concentrated form. Non-conjugated Ig is eluted first, followed by various "mers" (e.g., first by a "1-mer", an Ig conjugated to one A-chain, followed by a "2-mer", an Ig conjugated to two A-chains, etc., up to a "4-mer").

The immunoconjugate so isolated may then if desired be concentrated, e.g. by ultrafiltration, and desalted on a suitable column such as Trisacryl or Sephadex. The desalted immunoconjugate is filtered through a 0.2μ filter. A preferred final concentration of the purified immunoconjugate for medical use is at least about 4 mg/ml, and recoveries on the order of at least about 50–60% are typically obtained with this procedure.

In an alternative embodiment of the invention, a modified hydrophobic gel is provided for a "fast flow" chromatographic separation and purification step. The gel is either Phenyl Sepharose or TSK Phenyl-5PW, preferably Phenyl Sepharose, modified so as to contain only half the number of phenyl groups normally present. Such a modified gel is less hydrophobic, and thus does not bind the conjugate or Ig quite as strongly. Unconjugated Ig is removed with the first column volume of phosphate buffer/salt solution, as described above, and immunoconjugate is removed, typically, with a second column volume of phosphate buffer containing 10–60 vol. % of an organic solvent. In this procedure, the concentration of sodium chloride or ammonium sulfate in the first column volume of eluant, depending on the modified gel selected as above, is about 1.5M. Immunoconjugate is removed in this manner at a concentration of at least about 4 mg/ml, obviating the necessity for a concentration step following removal from the column.

It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples which follow are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

EXAMPLE 1

Monoclonal antibodies (designated MAB260F9) of the IgG class were obtained in P_iEDTA (0.10M $NaPO_4$, 1 mM EDTA, pH 8.0) at a concentration of 33.18 mg/ml. Purification was effected using a DEAE Sepharose (Pharmacia) column and ultrafiltration (0.2μ). The Ig was assayed for free thiols using dithionitrobenzoic acid (DTNB) and uv spectrometry to monitor released TNB groups, and it was determined that no free thiol groups were present in the Ig solution. Derivatization with DTNB and iminothiolane (IT) in preparation for coupling to the free thiol or ricin A was then accomplished by adding 363 μl of 1 mM DTNB and 525 μl 10 mM IT to the initial 8.71 ml of Ig. The reaction

temperature was maintained at about 0° C. and derivatization was allowed to proceed overnight, i.e. for about 24 hours. The derivatized conjugate was then desalted on a Trisacryl GF-05 column (LKB, Bromma, Sweden) buffered to a pH of 8.0 with P_iEDTA and using a flow rate of about 25 ml/hr.

Soluble recombinant ricin A chain (srRTA) was obtained by the method set forth in co-pending U.S. Patent Application Ser. No. 837,583. The srRTA was provided at an initial concentration of 10 mg/ml in P_iEDTA with 0.1% β-mercaptoethanol (BME) added. Contaminating particulate matter was removed by centrifugation of about 1000 rpm and desalting on a Trisacryl GF-05 column using P_iEDTA and a flow rate of about 25 ml/hr. A free thiol assay was run as described above, and it was determined that approximately 0.73 free thiols were present per molecule of srRTA.

Conjugation was accomplished by adding about 5 ml glycerol to the about 21.1 ml of desalted srRTA, followed by about 10.9 ml of Ig-TNB-IT complex prepared above. The reaction was allowed to proceed at about 25° C. for two hours, at which time it was presumed that conjugation was complete. A free thoil assay at this point gave a 96% conjugation efficiency.

The crude conjugate mixture so obtained was loaded onto a 950-ml Sephacryl S-300 column to remove unreacted srRTA and high molecular weight aggregates. The column was preequilibrated with a sodium chloride/phosphate buffer (pH 6.5; 0.1M Na_2PO_4; 1M NaCl; 1 mM EDTA). The conjugate was eluted with the buffer at a flow rate of about 40 ml/hr.

The resulting mixture, containing unconjugated Ig as well as various Ig/srRTA conjugates, was then loaded onto a 70 ml Phenyl Sepharose CL-4B column preequilibrated with the phosphate buffer of the preceding step. The initial conjugate pool was about 262 mg in 126 ml solution. Initial elution of the unreacted Ig was accomplished with a column volume of a 1M NaCl solution at a flow rate of about 20 ml/hr. Various "mers" of the immunoconjugate were then eluted as NaCl solutions of decreasing concentration were applied to the column (gradually decreasing from 1M to 0M), these NaCl solutions also containing increasing amounts of glycerol (gradually increasing from 0 (vol.)% to 60 (vol.)%, beginning with a 1:1 Ig:srRTA conjugate ("1-mer") and ultimately yielding a 1:4 Ig:srRTA conjugate ("4-mer"). The mixture was concentrated by ultrafiltration to about 4 mg/ml, desalted on a Trisacryl column as above, and filtered using a 0.2μ filter. Distribution and purity of the final immunoconjugate preparation was assayed by SDS polyacrylamide gel electrophoresis at: 44.0% 1-mer; 30.8% 2-mer; 10.7% 3-mer; 2.7% 4-mer. Cytoxicity as measured by $TCID_{50}$ (MCF-7 cells): 0.004 nM (minus lactose).

EXAMPLE 2

Immunoconjugate purification using the modified "fast flow" hydrophobic column: MAB260F9 antibodies and srRTA were obtained, purified and conjugated as in Example 1. The conjugate mixture was desalted using a Trisacryl column as described in Example 1, and applied to a hydrophobic gel column as follows.

The hydrophobic gel used in this example was Phenyl Sepharose Cl-4B modified by the manufacturer so as to reduce the standard number of phenyl groups by about 50%. The column (d1 cm; vol.3.14 ml) was equilibrated with 10 column volumes of P_i EDTA solution (0.1M Na_2PO_4, 1 mM EDTA) also containing 1.5M NaCl at a pH of about 8.0. The flow rate was set to about 0.13 ml/min and two eluting solutions were prepared: (A) 100 mM Na_2PO_4, pH 8.0, 1 mM EDTA, 1.5M NaCl; and (B) 100 mM Na_2PO_4, pH 8.0, 60 (vol.) % glycerol. The conjugate mixture was loaded onto the column, and unconjugated Ig was initially removed with solution (A) followed by removal of conjugate with solution (B). The column was then rinsed with 1 column volume of solution (B) to ensure complete removal of immunoconjugate.

EXAMPLE 3

Purification of a TNF Immunoconjugate

Purification of TNF Mutein

E. coli cells containing plasmid pAW731 were grown in a suitable growth medium for *E. coli* and were induced to produce TNF. The *E. coli* strain carrying pAW731 has been described in U.S. patent application Ser. No. 753,717, filed July 10, 1985, assigned to the same assignee as the present invention and incorporated herein by reference. The TNF produced by the strain had a single cysteine residue. After induction, the cells were removed from the medium and frozen. The cells were thawed, suspended in 100 ml 0.1M Tris, pH 8, 1 mM EDTA, and sonicated for 30 minutes.

The sonicated cells were centrifuged for 40 min at 12,000 g. The supernatant was removed, adjusted to 0.1M NaCl, and loaded onto a Phenyl Sepharose column previously equilibrated with 0.1M NaCl. The TNF eluted from the column in the flow through, and was dialysed against 0.1M Tris at pH 8.5, 1 mM EDTA. The dialysis retentate was loaded on a DEAE Sepharose column equilibrated with 0.01 M Tris, pH 8.5, 1 mM EDTA, and the TNF was eluted with 0.1M Tris, pH 8.5. The first protein fraction consisted of 95% pure TNF.

Murine monoclonal antibody 317G5 is described in U.S. patent application Ser. No. 690,750, filed Nov. 11, 1985, assigned to the same assignee as the present invention and herein incorporated by reference. 317G5 was derivatized with SPDP as described in U.S. patent application Ser. No. 690,750. Briefly, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) was added in a 20-fold molar excess to antibody. Following a 30 minute incubation at room temperature, the unreacted SPDP was removed by dialysis against PBS.

Conjugation

The SPDP-treated antibody was conjugated with TNF. Immediately prior to conjugation, the TNF was reduced with 50 mM dithiothreitol, then desalted on a column of chromatographic resin containing agarose, dextran and/or acrylamide to remove DTT from the protein. Reduced TNF was added in the three to five fold molar excess to the deriatized antibody, and the reaction was allowed to run overnight at 4° C.

The conjugate was loaded onto a Phenyl Sepharose column equilibrated with 2M NaCl and PBS. Free antibody was eluted off the column at 0.5M NaCl. The conjugate and free TNF were eluted off the column with PBS and 30% propylene glycol. Free TNF was separated from the conjugate by size exclusion chromatography using a S-200 Sepharose column.

We claim:

1. A method of purifying immunotoxin conjugates, comprising the steps of:

providing a conjugation mixture containing immunotoxin conjugate, unconjugated selective binding molecule and unconjugated toxin protein;

removing said unconjugated toxin protein from said mixture by gel filtration chromatography;

adding said mixture devoid of said unconjugated toxin protein to a hydrophobic gel chromatograph; and

removing said unconjugated binding molecule from said immunotoxin conjugate loaded on a hydrophobic gel with an eluting solution comprising an aqueous salt.

2. The method of claim **1**, wherein said step of removing unconjugated toxin on a sizing column precedes said step of removing the unconjugated binding molecule from toxin conjugate.

3. The method of claim **1**, wherein said step of removing the unconjugated binding molecule from the immunotoxin conjugate precedes the step of removing unconjugated toxin on a sizing column.

4. The method of claim **2**, wherein said toxin protein is a ribosome inactivating protein.

5. The method of claim **4**, wherein said toxin protein is ricin toxin A chain.

6. The method of claim **5**, wherein said ricin toxin A chain is recombinantly produced.

7. The method of claim **1**, wherein said toxin protein is tumor necrosis factor.

8. The method of claim **1**, wherein said binding molecule is selected from the group consisting of antibodies and fragments thereof that selectively bind to an epitope, hormones, cytokines, lymphokines and cell growth factors.

9. The method of claim **8**, wherein said binding molecule is selected from the group consisting of antibodies and fragments thereof that selectively bind to an epitope.

10. The method of claim **1**, wherein said eluting solution comprising an aqueous salt solution contains sodium chloride at a concentration of about 1.0M or less.

11. The method of claim **10**, wherein said aqueous salt solution is buffered to a pH ranging from about 6 to about 8.

12. The method of claim **1**, wherein said aqueous salt solution is in the range of about four and ten column volumes, each successively decreasing in salt concentration to about 0.5M.

13. The method of claim **1**, wherein said salt solution further comprises an organic solvent.

14. The method of claim **12**, wherein said column volumes of salt solution include an organic solvent increasing in amount up to about 60 volume percent.

15. The method of claim **14**, wherein said organic solvent is selected from the group consisting of glycerol, propylene glycol and ethanol.

16. A method of purifying immunotoxin conjugates, comprising the steps of:

providing a conjugation mixture containing immunoconjugate, unconjugated Ig and unconjugated ricin A chain;

removing said unconjugated ricin A chain from said conjugation mixture by gel filtration chromatography;

loading said mixture devoid of said unconjugated ricin A onto a colum filled with hydrophobic gel; and

removing said unconjugated Ig with at least one column volume of an aqueous salt solution.

17. The method of claim **16**, wherein said gel is provided with a number of phenyl groups at least sufficient to ensure hydrophobic retention of said conjugate mixture.

* * * * *

EXHIBIT 4

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Human Recombinant Alpha- and Gamma-Interferons Enhance the Cytotoxic Properties of Tumor Necrosis Factor on Human Melanoma

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Summary: Three short-term human melanoma cell lines were tested for sensitivity to human recombinant α-tumor necrosis factor (TNF) in a semisolid agar colony formation assay. Cells from three pigmented and one amelanotic strain displayed low sensitivity to TNF. The ID_{50} for the inhibition of melanoma colony formation ranged from 2,500 to 20,000 U/ml. We then tested the ability of human recombinant alpha-interferon (IFN-α) and gamma-interferon (IFN-γ) to interact with TNF to inhibit melanoma colony formation. Analysis of the TNF–IFN mixtures using the median effect method demonstrated that both IFNs interacted synergistically with TNF to inhibit melanoma colony formation. On a unit basis, IFN-γ was more active with TNF than IFN-α. The addition of the second interferon to the mixture enhanced the ability of TNF to promote the cytolysis of human melanoma cells. The enhanced killing effect seen with the combination of IFN-α, IFN-γ, and TNF suggests an interesting strategy for the treatment of human melanoma. **Key Words:** Interferon—Melanoma—Tumor necrosis factor.

Tumor necrosis factor (TNF) is a substance produced by macrophages in response to such stimuli as bacillus Calmette-Guerin and endotoxin (1,2). TNF can cause tumor cytolysis in vitro and in vivo (3–5), although the mechanism of action is not clearly understood. TNF has shown activity against various tumor cells including human tumor-burdened nude mice (6). Gamma-interferon (IFN-γ) and TNF in combination produce variable effects on tumor cells, ranging from synergistic antiproliferative (7,8) to cytotoxic responses (9,10).

We examined the ability of human recombinant alpha-interferon (IFN-α) and IFN-γ to interact with human recombinant TNF. The combination data were

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modeled using the median effect method (11). We found that either IFN sensitizes human melanoma cells to the cytotoxic properties of TNF.

MATERIALS AND METHODS

Human recombinant clone A alpha and gamma IFN were generous gifts from Hoffmann LaRoche Inc., Nutley, NJ, U.S.A. Human recombinant α-TNF was obtained from Genentech Inc., So. San Francisco, CA, U.S.A.

Human melanoma cell strains C8146C, C8161, and C83-2CY have been characterized (12,13). The cells were maintained in monolayer in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine (0.8 μg/ml), and gentamicin (10 μg/ml). All experiments that assessed colony formation with short-term human melanoma cell strains were done on cells subcultured less than seven times from the isolation from the original patient cells.

Soft Agar Assay

Human melanoma cells were plated in soft agar as previously described (12) and counted on day 14 by the FASII colony counter (14). Cell concentrations chosen for plating were those in the midrange cell dose, which produce a linear relationship between the number of cells plated and number of colonies formed (15).

Viability

Melanoma cells were plated in soft agar and were tested for viability on day 5 using a modification (13,16) of the 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) vital stain method (17). The dye was added on day 5, and the viable cells and growth units were scored on day 7.

Median Effect Analysis of Dose–Effect Curves

The colony-forming assay data were analyzed by the median effect method (11): fraction affected/fraction unaffected = $(\text{dose}/Dm)^m$, where Dm is the ID_{50} dose. The initial doses for the TNF–IFN combinations were chosen from the single-agent dose–response curves. If needed, these doses were changed in later experiments according to the outcome. Each concentration was done in triplicate, and each individual determination was used as a datum point. All dose–effect curves had linear regression coefficients greater than 0.9. An average combination index was determined. An average combination index of 1 denotes additivity, <1 synergism, and >1 antagonism.

RESULTS

The sensitivity of short-term human melanoma cell lines to recombinant TNF was tested in the soft agar assay. Dose–response curves were generated for each melanoma cell line. The colony-forming units in these melanoma cell lines displayed low sensitivity to TNF (Table 1). For the three pigmented human melanomas (C8146C, C82-7A, and C83-2CY), the ID_{50} ranged from 2,500 to 20,000

TABLE 1. *Sensitivity of short-term human melanoma colony-forming cells to treatment by recombinant α-tumor necrosis factor*

Human melanoma	ID_{50} (U/ml)
C8146C	2,500
C8161	7,500
C82-7A	20,000
C83-2CY	10,000

U/ml. A TNF dose of 7,500 U/ml was needed with the amelanotic human melanoma C8161 to reduce colony formation by 50%.

The recombinant IFNs were tested with TNF to determine if the combinations would promote a synergistic inhibition on human melanoma colony formation. Dose–response curves for each IFN, TNF, and IFN combination were developed. IFN-α and TNF in combination synergistically inhibited melanoma colony formation (Table 2). The average combination index for the two human melanoma cell lines C8161 and C83-2CY was 0.6 and 0.7, respectively. The IFN-γ–TNF mixture generated a stronger inhibition on human melanoma colony formation than did the IFN-α–TNF combination. The IFN-γ–TNF mixture displayed an average combination index that was <0.07 (Table 2). This combination index value is indicative of very strong synergism. Based on a unit basis, the TNF response in combination with IFN-γ can be up to 15 times stronger in inhibiting human melanoma colony formation than the one obtained with IFN-α.

As both IFNs combined with TNF produced a synergistic inhibition of melanoma colony formation, we explored whether or not a further synergism could be achieved by combining both IFN-α and IFN-γ with TNF. Figure 1 displays the dose–response curves for IFN-α and IFN-γ, which ranged between 100 and 1,000 U/ml. Higher concentrations of TNF were needed to obtain colony formation inhibition. Combining IFN-α, IFN-γ, and TNF in a 1:1:1 ratio resulted in a 1–2 log shift in the dose–response curves. For example, 33 U each of TNF, IFN-α, and IFN-γ in combination caused an 80% reduction in melanoma colony formation. This was strong synergistic interaction, as 33 U of either agent alone had no effect on melanoma colony formation. Similar results were obtained with the C8161 human melanoma line.

TABLE 2. *Analysis of the IFN–TNF combination interactions in the melanoma colony-forming assay*

Melanoma	Average combination index	Correlation coefficient	Result
C8161			
IFN-α + TNF	0.60	0.939	Synergism
IFN-γ + TNF	0.04	0.924	Very strong synergism
C8146C			
IFN-γ + TNF	0.06	0.985	Very strong synergism
C83-2CY			
IFN-α + TNF	0.73	0.996	Synergism
IFN-γ + TNF	0.02	0.916	Very strong synergism

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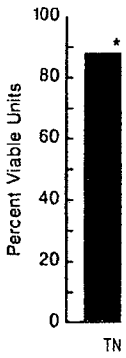

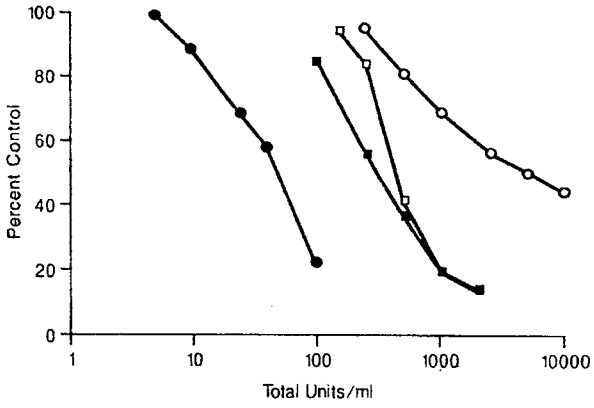

FIG. 1. Dose-dependent inhibition of C83-2CY human melanoma colony formation by IFN-α (■), IFN-γ (□), TNF (○), and IFN-α, IFN-γ, and TNF in combination at a 1:1:1 ratio (●).

Does this syngerism between the IFNs and TNF manifest itself in the cytolysis of melanoma cells? C83-2CY cells were plated in soft agar, and groups of plates were exposed to 500 U of either IFN alone, TNF alone, IFN-α + IFN-γ, and combinations of IFN–TNF. On day 5, INT stain was added to the plates, and on day 7, the red-colored viable single cell and multi-cell units were counted. Day 5 was chosen because from monolayer studies, a 48–72-h exposure was needed for cytolysis. As single agents, TNF and IFN-α did not appreciably reduce melanoma viability (Fig. 2). IFN-γ reduced the number of red units by 25%. The IFN-α + IFN-γ combination resulted in a 29% reduction in viable melanoma units, which was not significantly different from the IFN-γ treatment (not shown). A significant enhanced reduction in viability was observed with the IFN–TNF combinations. The IFN-α–TNF combination reduced the viability of the culture by 40%. The IFN-γ–TNF combination produced the largest reduction in melanoma viability (55%). These reductions in viability were greater than would be predicted for an additive interaction. The [IFN-α, IFN-γ, TNF] combination resulted in a further synergistic reduction in melanoma viability. Ninety percent of the plated melanoma cells were killed by the simultaneous addition of IFN-α, IFN-γ, and TNF.

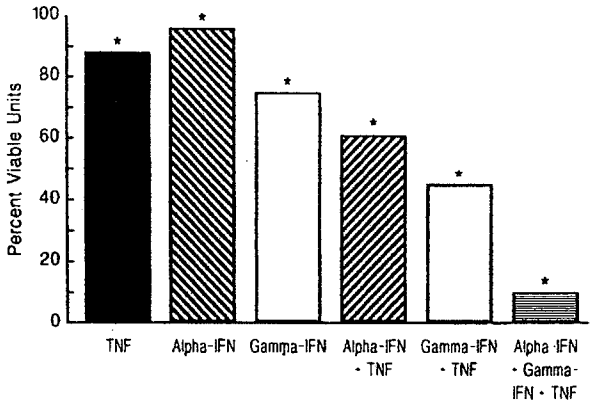

FIG. 2. Effect of TNF, IFNs, and TNF–IFN combinations on the viability of C83-2CY human melanoma cells plated in soft agar. A quantity of 500 U/ml of each agent was added to the various groups. The standard error is represented by (*).

DISCUSSION

Tumor necrosis factor is a cytotoxic protein that has promise as an antitumor agent. The question has been raised by recent data of whether TNF works in vivo by direct cytolysis of tumor cells, immune modulation, or by direct action on tumor vasculature (18,19). In this study, pure human melanoma cells were tested and found to be insensitive to low doses of TNF (1–1,000 U/ml). Low doses of TNF (1–100 U/ml) cause cytolysis with sensitive cell lines BT-20 and MCF-7 (breast cancer) and ME (cervical cancer) (20). Either IFN-α or IFN-γ interacted synergistically with TNF to inhibit colony formation. The IFN-γ–TNF combination generated the strongest synergistic interaction, which resulted in the cytolysis of the human melanoma cells. The IFN-γ–TNF combination is active on human breast, colon, and cervix carcinomas, but not leukemias, lymphomas, or normal cells (10). IFN-α and IFN-γ act through unique receptors (21–23), and in combination with TNF, they may display different tumor-specific activity profiles. The IFN-α–TNF combination has not been extensively explored. Our observation that IFN-α promotes the cytolytic properties of TNF on human melanoma cells indicates that this combination should be tested on other tumor types.

In combination, α/β and γ-IFN generate synergistic antiproliferative effects (24–27). We tested whether or not this synergism between the interferons extended to sensitizing human melanoma cells to the cytolytic effects of TNF. In our study, a pronounced 1–2 log dose shift was observed on melanoma colony formation when IFN-α, IFN-γ, and TNF were used in combination. The addition of the second interferon to the mixture enhanced the ability of TNF to promote the cytolysis of human melanoma cells. The enhanced killing effect seen with the IFN-α–IFN-γ–TNF mixture suggests an interesting strategy for the treatment of human melanoma.

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EXHIBIT 5

US005135736A

United States Patent [19]

Anderson et al.

[11] Patent Number: 5,135,736

[45] Date of Patent: Aug. 4, 1992

[54] COVALENTLY-LINKED COMPLEXES AND METHODS FOR ENHANCED CYTOTOXICITY AND IMAGING

[75] Inventors: David C. Anderson, Seattle; A. C. Morgan, Jr., Edmonds; Paul G. Abrams, Seattle, all of Wash.

[73] Assignee: NeoRx Corporation, Seattle, Wash.

[21] Appl. No.: 232,337

[22] Filed: Aug. 15, 1988

[51] Int. Cl.[5] A61K 39/44; A61K 49/02; A61K 49/04

[52] U.S. Cl. 424/1.1; 424/9; 424/85.91; 424/94.3; 514/8; 514/12; 514/21; 530/395; 530/391.7; 530/389.7; 530/288.15; 530/388.8; 530/387.1; 530/391.3; 435/188

[58] Field of Search 530/390, 391, 388, 395; 424/1.1, 9, 85.91, 94.3; 514/12, 21, 8; 435/188

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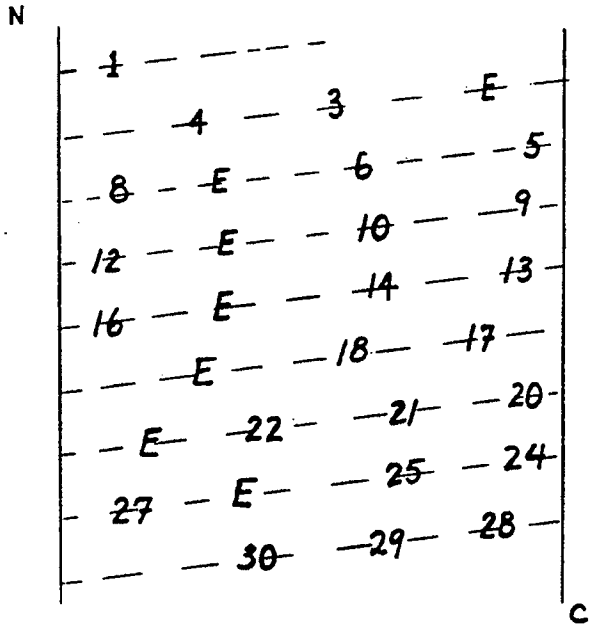
Assistant Examiner—Kay K. Kim
Attorney, Agent, or Firm—Stoel Rives Boley Jones & Grey

[57] ABSTRACT

Covalently-linked complexes (CLCs) for targeting a defined population of cells, comprising a targeting protein or peptide; a cytotoxic agent; and an enhancing moiety, wherein the enhancing moiety is capable of promoting CLC-membrane interaction are disclosed. Methods for using the claimed CLCs to obtain enhanced in vivo cytotoxicity and enhanced in vivo imaging are also described.

13 Claims, 1 Drawing Sheet



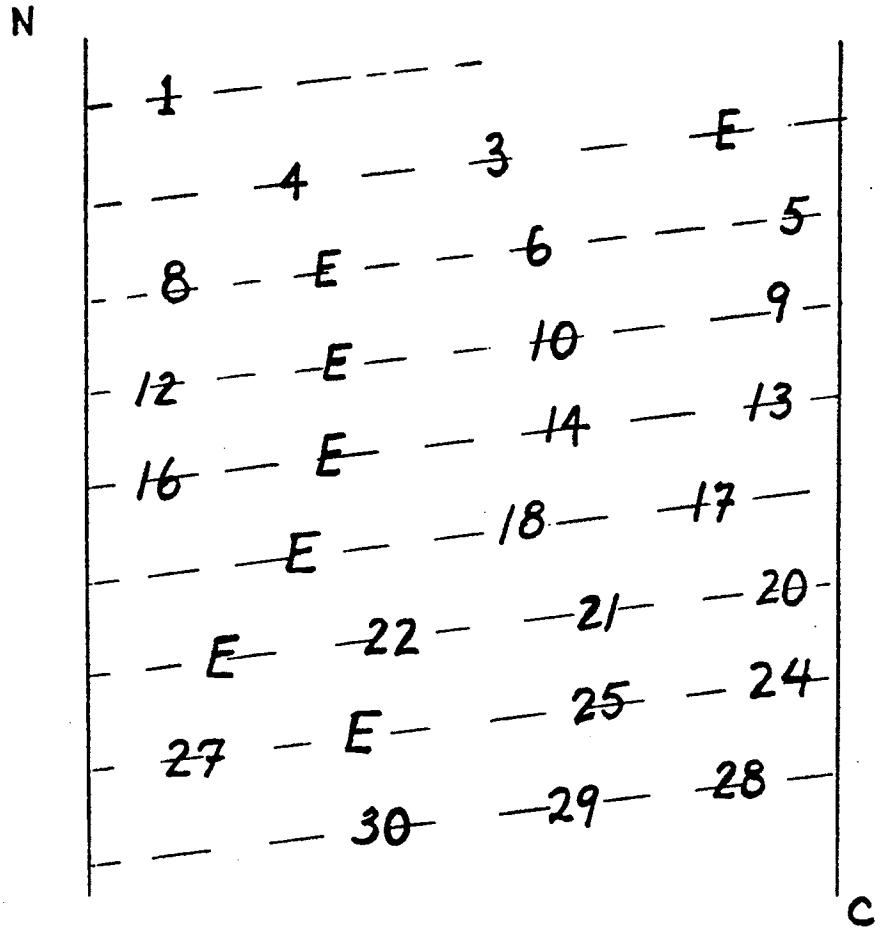


Figure 1

COVALENTLY-LINKED COMPLEXES AND METHODS FOR ENHANCED CYTOTOXICITY AND IMAGING

TECHNICAL FIELD

The present invention relates to covalently-linked complexes (CLC) having enhanced diagnostic or therapeutic properties and methods of using these complexes. The CLC of the present invention has three components: (1) a targeting protein or peptide; (2) a cytotoxic agent, such as a radioisotope, a drug or a toxin; and (3) one or more enhancing moieties capable of promoting CLC-target cell membrane interaction.

BACKGROUND OF THE INVENTION

Immunoconjugates consisting of antibody joined to a cytotoxic agent have been used in attempts to achieve selective killing of particular target cells, such as tumor cells. In theory, immunoconjugates or targeting protein conjugates should effect specific cellular cytotoxicity. In practice, however, in vivo administration of immunoconjugates has proven less efficacious than anticipated.

Several disadvantages related to retention, internalization and translocation of immunoconjugates have been identified. For instance, optimal retention of isotope-antibody fragment conjugates within tumor tissue after in vivo administration has not been demonstrated. Additional problems associated with target cell internalization and translocation of immunoconjugates have been recognized, particularly in regards to translocation and internalization of A-chain (derived from plant o r bacterial toxin) immunoconjugates.

Thus, there is a need in the art for improved: (1) retention of targeting protein conjugates (especially antibody fragment conjugates) at target cell plasma membranes; (2) internalization of targeting protein conjugates into target cell endosomic vesicles; and (3) translocation of targeting protein conjugates across target cell endosomic vesicular membranes into the cytoplasm. Enhancement of the interaction of targeting protein conjugates with plasma membranes and/or internal membranes of target cells may improve the cytotoxicity of targeting protein conjugates administered in vivo. The present invention fulfills this need and further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention describes a covalently-linked complex (CLC) for targeting a defined population of cells, comprising a targeting protein or a targeting peptide; a cytotoxic agent; and an enhancing moiety, wherein the enhancing moiety is capable of promoting CLC-membrane interaction.

A method for enhancing in vivo cytotoxicity of a targeting protein conjugate comprising administering to a tumor-bearing patient a therapeutically effective amount of the covalently-linked complex of the present invention is also disclosed.

In addition, a method for enhanced in vivo imaging of a tumor comprising administering to a tumor-bearing patient a diagnostically effective amount of the claimed covalently-linked complex is disclosed.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates a helical net structure representing an advantageous spatial arrangement of amino acids present in a translocating peptide of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to set forth definitions of certain terms to be used within the disclosure.

Targeting protein or targeting peptide: A protein or peptide that binds to a defined population of cells. The targeting protein or peptide may bind a receptor, an enzymatic substrate, an antigenic determinant, or other binding site present on the target cell population. Hereinafter, the term "targeting protein" will be inclusive of both targeting proteins and targeting peptides.

Translocating peptide: A peptide capable of insertion into membranes at acidic pH (typically pH 5.0–5.5).

Anchoring peptide: A peptide capable of insertion into membranes at physiological pH (typically pH 6.8–7.5).

Accessory peptide: A peptide or non-peptide molecule that serves as a substrate for target cell enzymes, and promotes membrane retention or translocation of an anchoring and/or translocating peptide.

Fusion protein: A hybrid protein generated by means of recombinant DNA technology. A fusion protein is translated from messenger RNA as one continuous polypeptide chain, with the protein or peptide components joined together by peptide bonds.

Conjugate: A two-component hybrid molecule wherein the components are joined by a covalent chemical linkage.

Targeting protein conjugate: A conjugate wherein one component is antibody (i.e., an immunoconjugate) or, more generally, a targeting protein. Typically, the second component of a targeting protein conjugate is a cytotoxic agent, such as a drug, a toxin or a radionuclide. In contrast to fusion proteins, recombinant DNA methods are not involved in the covalent linkage of targeting protein conjugate components.

Covalently-linked complex (CLC): A three-component complex comprising (1) a targeting protein; (2) a cytotoxic agent; and (3) an enhancing moiety; wherein the three components of the CLC are joined together by covalent bonds.

Enhancing moiety: A moiety capable of promoting membrane interaction. Enhancing moieties of the present invention include translocating peptides, anchoring peptides, accessory peptides and organic membrane intercalators. In addition, an enhancing moiety may be fused to one or more components of a fusion protein. One or more enhancing moieties may be covalently attached to a targeting protein conjugate to form a CLC having enhanced membrane interactive characteristics.

In general, three levels of targeting protein conjugate-membrane interaction have been identified that may be important for optimal in vivo diagnostic or therapeutic efficacy: (1) binding of the conjugate to the target cell plasma membrane; (2) internalization of the conjugate into endosomic vesicles; and (3) translocation of the conjugate from endosomic vesicles into the cytoplasm, which gives a targeting protein conjugate access to cytoplasmic or nuclear target sites. If any one of these targeting protein conjugate-membrane interactive steps becomes rate-limiting, targeting protein conjugate potency may be diminished.

Optimization of the three levels of target cell membrane interaction noted above (i.e., retention, translocation, internalization) may enhance the cytotoxicity of targeting protein conjugates. Different types of targeting protein conjugates (for instance, targeting protein conjugated to either a drug, toxin or radioisotope) may require different levels of targeting protein conjugate-membrane interaction in order to achieve optimal cytotoxicity in vivo.

More specifically, radioisotope-targeting protein conjugates require binding and prolonged retention of the conjugate, either within the tumor or at the tumor cell plasma membrane, for maximal cytotoxic efficacy. Drug-targeting protein conjugates that are active at the plasma membrane may require (1) binding of the targeting protein conjugate at the plasma membrane, and (2) expression of cytolytic activity at the plasma membrane. Drug-targeting protein conjugates that are not active at the target cell plasma membrane additionally require internalization of the drug for cytotoxicity. Drug conjugates of this latter type and toxin-targeting protein conjugates require three levels of membrane interaction for cytotoxicity: (1) binding of the targeting protein conjugate at the plasma membrane; (2) internalization of the conjugate within the target cell; and (3) translocation of the conjugate from endosomic vesicles into the cytoplasm.

The "targeting protein" component of the covalently-linked complex (CLC) of the present invention directs a covalently-attached cytotoxic agent to a target cell population, such as tumor cells. Preferred targeting proteins useful in this regard include antibody and antibody fragments; peptides, such as bombesin, gastrin-releasing peptide, RGD peptide, substance P, neuromedin-B, neuromedin-C, and metenkephalin; and hormores, such as EGF, α- and β-TGF, estradiol, neurotensin, melanocyte stimulating hormone, follicle stimulating hormone, luteinizing hormone, and human growth hormone. Biotin, avidin, proteins corresponding to known cell surface receptors (including low density lipoproteins, transferrin and insulin), fibrinolytic enzymes, and biological response modifiers (including interleukin, interferon, erythropoietin and colony-stimulating factor) are also preferred targeting proteins. Analogs of the above-listed targeting proteins that retain the capacity to bind to a defined target cell population may also be used within the claimed invention. In addition, synthetic targeting proteins and peptides may be designed.

Monoclonal antibodies have precise specificity for a particular epitope present on a target cell population. When a cytotoxic agent, such as a drug, toxin or radioisotope, is conjugated to a monoclonal antibody, increased amounts of the cytotoxic agent may be administered in vivo (as compared to the unconjugated form of the cytotoxic agent), due to the selective targeting properties of the monoclonal antibody component of the conjugate.

Types of cytotoxic agents useful herein include toxins, drugs and radionuclides. Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only the toxin A chain to a targeting protein. However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the toxin A chain-targeting protein conjugate, as compared to the corresponding holotoxin-targeting protein conjugate.

One possible explanation for the decreased potency of A chain-targeting protein conjugates is that B chain is required for translocation of the A chain across endosomic membranes into the target cell cytoplasm. In the absence of translocation, the targeting protein conjugate remains in the interior of an endosome, and is ultimately transported to a lysosome. Within the lysosome, the targeting protein conjugate is degraded, and thus the A chain cytotoxic agent fails to reach its cytoplasmic target site. The decreased potency associated with toxin A chain-targeting protein conjugates also accompanies the use of ribosomal inactivating protein-targeting protein conjugates. Ribosomal inactivating proteins (RIPs) are naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability.

Within the present invention, preferred toxins include holotoxins, such as abrin, ricin, modeccin, Pseudomonas exotoxin A, Diphtheria toxin, pertussis toxin and Shiga toxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of Pseudomonas exotoxin A, Diphtheria toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin and snake venom peptides.

Preferred drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 6-thioguanine, cytarabine, cyclophosphamide and cisplatinum, as well as other conventional chemotherapeutics as described in *Cancer: Principles and Practice of Oncology*, 2d ed., V. T. DeVita, Jr., S. Hellman, S. A. Rosenberg, J. B. Lippincott Co., Philadelphia, Pa., 1985, Chapter 14. A particularly preferred drug within the present invention is a trichothecene.

Experimental drugs, such as mercaptopurine, N-methylformamide, 2-amino-1,3,4-thiadiazole, melphalan, hexamethylmelamine, gallium nitrate, 3% thymidine, dichloromethotrexate, mitoguazone, suramin, bromodeoxyuridine, iododeoxyuridine, semustine, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea, N,N'-hexamethylene-bis-acetamide, azacitidine, dibromodulcitol, Erwinia asparaginase, ifosfamide, 2-mercaptoethane sulfonate, teniposide, taxol, 3-deazauridine, soluble Baker's antifol, homoharringtonine, cyclocytidine, acivicin, ICRF-187, spiromustine, levamisole, chlorozotocin, aziridinyl benzoquinone, spirogermanium, aclarubicin, pentostatin, PALA, protein conjugates, only plasma membrane interaction is required, but effective cytotoxicity requires prolonged retention of the conjugate at the target membrane.

The present invention discloses compositions and methods that promote interaction(s) of targeting protein conjugates with various target cell membranes. More specifically, biochemical linkage of a targeting protein conjugate and one or more enhancing moieties capable of promoting membrane interaction (or construction of an analogous recombinant fusion protein) results in a "covalently-linked complex" (CLC) having improved membrane interactive properties.

Enhancing moieties useful within the present invention may be subdivided into several categories. The first category of enhancing moieties is designated "translocating peptides," and includes pH-dependent mem-

brane-binding peptides. The second category is designated "anchoring peptides," and includes membrane soluble peptide sequences and analogs thereof. Anchoring peptides are capable of binding to target cell membranes at physiologic pHs. A third category, "accessory peptides," may be used in conjunction with translocating or anchoring peptides to enhance membrane retention and/or translocation. A fourth category of enhancing moieties includes membrane permeation enhancers, designated "organic membrane intercalators," such as fatty acids and analogs thereof, bile salts, membrane anesthetics, phospholipids, medium chain glycerides and fusidic acid.

In an alternative embodiment, one or more enhancing moieties may be included in a fusion protein. For generation of a fusion protein that contains an enhancing moiety, a first DNA sequence (corresponding to a targeting protein, a cytotoxic agent or an enhancing moiety) is joined at the DNA level through recombinant DNA technology to a similar or dissimilar second (third, fourth, etc.) DNA sequence. The resultant fused DNA carboplatin, amsacrine, caracemide, iproplatin, misonidazole, dihydro-5-azacytidine, 4'-decoxy-doxorubicin, menogaril, triciribine phosphate, fazarabine, tiazofurin, teroxirone, ethiofos, N-(2-hydroxyethyl)-2-nitro-1H-imidazole-1-acetamide, mitroxantrone, acodazole, amonafide, fludarabine phosphate, pibenzimol, didemnin B, merbarone, dihydrolenperone, flavone-8-acetic acid, oxantrazole, ipomeanol, trimetrexate, deoxyspergualin, echinomycin, and dideoxycytidine (see *NCI Investigational Drugs, Pharmaceutical Data* 1987, NIH Publication No. 88-2141, Revised November 1987) are also preferred.

Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, X-ray emitters and fluorescenceemitters, with beta- or alpha-emitters preferred for therapeutic use. Radionuclides are well-known in the art and include ^{123}I, ^{125}I, ^{130}I, ^{131}I, ^{133}I, ^{135}I, ^{47}Sc, ^{72}As, ^{72}Se, ^{90}Y, ^{88}Y, ^{97}Ru, ^{100}Pd, ^{101m}Rh, ^{119}Sb, ^{128}Ba, ^{197}Hg, ^{211}At, ^{212}Bi, ^{212}Pb, ^{109}Pd, ^{111}In, ^{67}Ga, ^{68}Ga, ^{67}Cu, ^{75}Br, ^{76}Br, ^{77}Br, ^{99m}Tc, ^{11}C, ^{13}N, ^{15}O and ^{18}F. Preferred therapeutic radionuclides include ^{188}Re, ^{186}Re, ^{203}Pb, ^{212}Pb, ^{212}Bi, ^{109}Pd, ^{64}Cu, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au and ^{199}Ag.

As noted above, with toxin-targeting protein conjugates, the limiting membrane interactive events appear to be the rate of internalization and the rate of translocation. With respect to drug-targeting protein conjugates that are not active at the plasma membrane, internalization of the targeting protein conjugate is required, with release of the drug from endosomic vesicles before the conjugate reaches the lysosome (i.e., is degraded). With drug-targeting protein conjugates that are active at the plasma membrane, internalization is not required, but a strong, prolonged interaction of the drug conjugate at the plasma membrane is important for cytotoxic efficacy. For radionuclide-targeting sequences are transcribed and translated into a hybrid fusion protein. When an enhancing moiety is incorporated into a fusion protein, the resultant fusion protein possesses improved membrane interactive properties.

In general, according to the present invention, the targeting protein component of a covalently-linked complex recognizes a binding site at the target cell membrane surface. A primary target cell interaction mediated by the targeting protein component of the CLC is followed by a secondary interaction of the enhancing moiety component with the plasma membrane. This secondary interaction between enhancing moiety and membrane stabilizes the targeting protein at the membrane surface. In instances where an antibody (or antibody fragment) is the targeting protein, interaction of enhancing moiety and target cell membrane may also increase the affinity of an antibody for its antigen.

Translocating Peptides

The first category of enhancing moiety consists of translocating peptides, which exhibit pH-dependent membrane binding. When a translocating peptide assumes a helical conformation at an acidic pH, the translocating peptide acquires the property of amphiphilicity, e.g., it has both hydrophobic and hydrophilic interfaces. More specifically, within a pH range of approximately 5.0–5.5, a translocating peptide forms an alpha-helical, amphiphilic structure that facilitates insertion of the translocating peptide into a target membrane. An alphahelix-inducing acidic pH environment may be found, for example, in the low pH environment present within cellular endosomes.

In aqueous solution at physiological pH, a translocating peptide is unfolded (due to strong charge repulsion among charged amino acid side chains) and is unable to interact with membranes. Within the present invention, it may be advantageous to position amino acid residues within the translocating peptide sequence so that charged amino acid side chains will stack one above the other when the peptide folds into an amphiphilic alpha helix at reduced pH. FIG. 1 represents a helical net display that illustrates an advantageous spatial arrangement of the charged side chains.

Charged amino acids capable of stacking within a translocating peptide sequence include glutamate, aspartate and histidine. A preferred pH-dependent membrane-binding translocating peptide includes a high percentage of helix-forming residues, such as glutamate, methionine, alanine and leucine. In addition, a preferred translocating peptide sequence includes ionizable residues having pKa's within the range of pH 5–7, so that a sufficient uncharged membrane-binding domain will be present within the peptide at pH 5 to allow insertion into the target cell membrane.

A particularly preferred pH-dependent membrane-binding translocating peptide in this regard is aa1-aa2-aa3-EAALA(EALA)$_4$-EALEALAA-amide, which represents a modification of the peptide sequence of Subbarao et al. (*Biochemistry* 26: 2964, 1987). Within this peptide sequence, the first amino acid residue (aa1) is preferably a unique residue, such as cysteine or lysine, that facilitates chemical conjugation of the translocating peptide to a targeting protein conjugate. Amino acid residues 2–3 may be selected to modulate the affinity of the translocating peptide for different membranes. For instance, if both residues 2 and 3 are lys or arg, the translocating peptide will have the capacity to bind to membranes or patches of lipids having a negative surface charge. If residues 2–3 are neutral amino acids, the translocating peptide will insert into neutral membranes.

Yet other preferred translocating peptides include peptides of apo-lipoprotein A-1 and B; peptide toxins, such as melittin, bombolittin, delta hemolysin and the pardaxins; antibiotic peptides, such as alamethicin; peptide hormones, such as calcitonin, corticotrophin releasing factor, beta endorphin, glucagon, parathyroid hormone, pancreatic polypeptide; and peptides corre-

sponding to signal sequences of numerous secreted proteins. In addition, exemplary translocating peptides may be modified through attachment of substituents that enhance the alpha-helical character of the translocating peptide at acidic pH.

An example of a modified peptide having translocating activity at acidic pH is fully succinylated melittin. In this example, a peptide (melittin) that normally binds to membranes at physiological pH is converted to a pH-dependent translocating peptide through succinylation of lysines. Upon succinylation, the peptide displays an amphipathic character only at acidic pHs.

Within the present invention, translocating peptides may be designed and synthesized to provide enhanced membrane interaction(s). For instance, translocating peptides conforming to the helical net structure depicted in FIG. 1 may be generated. More specifically, in a translocating peptide designed according to FIG. 1, stacked glutamates may occupy positions in the helical net that are designated as "E"; the remaining amino acid residues may consist (entirely or predominantly) of strong neutral helix formers, such as methionine, alanine or leucine.

Insertion of a translocating peptide into a target cell membrane may be enhanced through stabilization of the amphiphilic alpha helix. Helix stabilization may be achieved: (1) by adding repeating "EALA" units to form a longer peptide; (2) by placing an amide at the C-terminus of the peptide, in order to counteract the helical dipole; (3) by polymerizing the peptide; (4) by substituting a neutral helix-former for one or more of the stacked glutamates; or (5) by attaching the peptide to a targeting protein conjugate through use of a longer crosslinking agent, in order to provide sufficient distance between the translocating peptide and the targeting protein conjugate to allow the peptide to contact and interact with the target cell membrane.

The amino acid sequence of a pH-dependent, membrane-binding translocating peptide of the claimed invention may be selected to include all L-amino acids or all D-amino acids having a side chain pKa from 5.0 to 9.0. D-amino acids may be advantageously used to form non-proteolyzable peptides, because the D-amino acids are not metabolized within the cell. Further, the translocating peptides of the present invention may include a combination of L- and D-amino acids, wherein D-amino acids are substituted for L-amino acids on either side of a proteolytic cleavage site. Yet another preferred noncleavable translocating peptide incorporates peptide bond analogs that are not susceptible to proteolytic cleavage by cellular enzymes.

The pH-dependent, membrane-binding translocating peptides of the present invention may be attached singly or may be polymerized. Chemical linkage of one or more translocating peptides (single, or polymerized) may be accomplished either: (a) by direct attachment of a translocating peptide to a targeting protein conjugate; (b) by disulfide, thioether, reduced or non-reduced Schiff base or peptide bond formation; or (c) by use of a peptide spacer. Preferred peptide spacers in this regard correspond to two or more amino acid residues that allow the translocating peptide to assume an alpha-helical conformation independent of its interaction with the targeting protein conjugate, and may allow sufficient distance for translocating peptide access to the cell surface from the peptide attachment site on the targeting protein.

Polymerization of translocating peptides may be accomplished by placing a cysteine residue at each end of a translocating peptide, followed by oxidation using dissolved oxygen or other mild oxidizing agent, such as oxidized glutathione. The average length of the polymerized translocating peptide may be controlled by varying the polymerization reaction conditions.

Mere membrane intercalation of a translocating peptide may be sufficient for translocation of the peptide across endosomic membranes. However, translocation may be improved by attaching to the translocating peptide moiety a substrate for intracellular enzymes (i.e., an accessory peptide, to be discussed in more detail in a following section). It is preferred that an accessory peptide be attached to a portion(s) of the translocating peptide that protrudes through the endosomic membrane to the cytoplasmic face.

Covalent linkage of an enhancing moiety and a targeting protein conjugate (forming a CLC) may provide enhanced retention of the conjugate (complex) at a target cell plasma membrane upon in vivo administration. In addition, a covalently-linked complex may exhibit more rapid and efficient internalization rates than the corresponding two-component targeting protein conjugate, due to secondary interaction(s) of the enhancing moiety with the plasma membrane. Inclusion of both anchoring and translocating peptides within a CLC may further facilitate initial binding and enhanced translocation of the CLC across endosomic membranes into the target cell cytoplasm.

Anchoring peptides

A second category of enhancing moiety suitable for use within the present invention consists of anchoring peptides. Typically, anchoring peptides contain membrane soluble peptide sequences which are highly apolar and characteristically form alpha helices when inserted into a membrane. When incorporated into a CLC, membrane insertion of the anchoring peptide component may help to secure the targeting protein conjugate component to a target cell membrane, and may further promote internalization of the targeting protein conjugate/CLC.

A model for anchoring peptide interaction with a plasma membrane is the opiate form of beta endorphin. Beta endorphin has one region that is responsible for receptor binding, and another region which can assume an amphiphilic helix (anchoring portion). The amphiphilic helix of beta endorphin is believed to be responsible for an initial membrane interaction, which is followed by diffusion of the hormone through the cell membrane. Diffusion through the membrane allows the receptor-binding region of the molecule to find its appropriate receptor (binding site). In theory, the reverse process may also occur —— the receptor binding portion (targeting portion) of beta endorphin interacts with its receptor, followed by alpha-helix formation and membrane insertion of the anchoring portion of the molecule.

Anchoring peptides suitable for use within the present invention may be (i) chemically synthesized; (ii) made by recombinant DNA technology; or (iii) isolated from viral fusion proteins or other proteins. Viral fusion peptides, such as those described by Gallaher (*Cell.* 50: 327–28, 1987), are exemplary of anchoring peptides of the claimed invention. Preferred viral fusion peptide sequences in this regard may be derived from viral proteins of measles virus, respiratory syncytial virus,

Sendai virus, murine mammary tumor virus, human or simian immunodeficiency virus, visna virus, or simian retrovirus. In addition, analogs of viral fusion peptides that retain the capacity to embed within a membrane may be suitable for use within the claimed invention.

AVGAIGAMFLGFLGAAGSTMGAASL represents yet another preferred anchoring peptide sequence that may be incorporated into a covalently-linked complex according to the present invention. An anchoring peptide sequence that includes one or more internal repeats of the sequence "-FLG-" or "-FLA-" or combinations thereof may also be preferred. For some therapeutic applications, the addition of one or more negatively charged residues to the anchoring peptide may be preferred. The additional negatively charged residues may decrease levels of non-specific binding mediated by the peptide domain of the CLC.

In a preferred embodiment, the anchoring peptide sequence includes an N terminal aa1-aa2-aa3 sequence, which is defined in the same manner as "aa1-aa2-aa3" of the pH-dependent, membrane-binding translocating peptides, as described above. In addition, variable length peptide spacers may be added to either terminus of the anchoring peptide sequence. The remainder of the anchoring peptide sequence includes amino acid residues capable of fusing with membranes or lipid bilayers.

In another preferred embodiment of the present invention, an anchoring peptide may be attached to the targeting protein conjugate component of a CLC by means of variable length crosslinking agents. In certain instances, longer crosslinker spacer arms between the enhancing moiety and the targeting protein conjugate are preferred. The span of a longer crosslinking agent permits an anchoring peptide to reach from the binding site of the targeting protein component to the target cell membrane.

In addition, aa2 and aa3 of an anchoring peptide sequence may be substituted with a peptide spacer consisting of 1–40 amino acids. The entire anchoring peptide plus spacer may be produced chemically in one synthetic reaction. In a preferred embodiment, the spacer does not assume a beta sheet or helical shape, and may be retained in an extended conformation at physiological pH by charge repulsion.

A preferred spacer in this regard is CDNDNDDNDDGGG. Alternatively, a preferred peptide spacer would include predominantly polar (charged or uncharged) residues to aid solubility and, for spacers having charged residues, only like charges. The sequence CRQRQRRQRRGGG is exemplary of a positively charged spacer. The peptide spacers of the present invention typically have a unique N-terminal residue (such as cys, lys, asp, or glu) useful for crosslinking to a targeting protein. The insertion of a peptide spacer provides greater distance between the targeting protein binding site and the anchoring peptide, thereby increasing the probability that the anchoring peptide will reach the target cell membrane and insert. For instance, a 10-mer peptide spacer, conformationally decoupled from a helix-forming anchoring peptide by insertion of three glycine residues, would span approximately 30–40 Å in an extended conformation. This type of peptide spacer may also be advantageously used with translocating peptides of the present invention. Alternatively, polymeric forms of anchoring peptides may be used to span the distance from a targeting protein binding site to the target membrane.

In instances where an anchoring peptide has a propensity for non-specific insertion into non-target cell membranes, it may be desirable to decrease the probability of membrane insertion of the anchoring peptide. Anchoring peptide insertion into a membrane could be made less probable (1) by shortening the anchoring peptide; (2) by including weaker neutral helix formers in non-glutamate positions within the peptide sequence (see FIG. 1); (3) by substituting aspartate for glutamate within the anchoring peptide sequence; (4) by synthesizing an anchoring peptide with a C terminal carboxylate group; or (5) by incorporating into the peptide sequence uncharged amino acids that are slightly more hydrophilic than residues of a strongly translocating/anchoring peptide. By implementing such peptide modifications, anchoring peptide dissolution in membranes would be predicted to occur only upon primary interaction of the targeting protein component with its binding site.

In one preferred embodiment, a virus-derived anchoring peptide sequence is covalently attached to a targeting protein conjugate, forming a covalently-linked complex. Antibody fragments, as well as intact antibody molecules, are preferred targeting proteins for anchoring peptide attachment.

Upon in vivo administration of a CLC, a primary interaction of the targeting protein component with its binding site is followed by a secondary interaction of the anchoring peptide component with the target cell plasma membrane. The anchoring peptide component of the CLC is solubilized within the membrane, thereby anchoring the targeting protein conjugate component into the target cell membrane. The anchoring peptide component may also act to enhance translocation of the CLC into the target cell.

Accessory peptides

A third category of enhancing moiety, the "accessory peptides," may be advantageously attached to the carboxy terminus of a translocating or anchoring peptide. An accessory peptide of the present invention may contain one or more amino acid residues. In one embodiment, an accessory peptide may provide a substrate for cellular phosphorylation (for instance, the accessory peptide may contain a tyrosine residue).

To exemplify the use of an accessory peptide within the claimed invention, a phosphorylatable accessory peptide is first covalently attached to the C-terminus of an anchoring peptide and then incorporated into a covalently-linked complex. The anchoring peptide component of the CLC intercalates into the target cell plasma membrane and, as a result, the accessory peptide is translocated across the membrane and protrudes into the cytoplasm of the target cell. On the cytoplasmic side of the plasma membrane, the accessory peptide is phosphorylated by cellular kinases at neutral pH. Once phosphorylated, the accessory peptide acts to irreversibly anchor the CLC into the membrane. Further, the accessory peptide may enhance the translocation of the CLC into the cell cytoplasm.

Preferred accessory peptides in this regard include kinase-substrate accessory peptides that incorporate serine. A kinase-substrate accessory peptide may be particularly advantageous for enhancement of CLC cytotoxicity of tumor target cells, which have increased levels of protein kinase activity for serines or tyrosines. Increased levels of kinase activity within tumor cells may be attributed to the presence of oncogene products,

such as H-ras, on the cytoplasmic side of tumor cell plasma membranes.

Suitable accessory peptides also include peptides that are kinase substrates and peptides that possess a single positive charge. The latter type of accessory peptide may form an ion pair with a "glutamate-like" residue of an attached or closely adjacent translocating peptide. In this regard, it may be desirable to replace an accessory peptide lysine and/or arginine residue(s) with histidine, in order to facilitate movement of a more neutral peptide through a target membrane at acidic pH.

Particularly preferred accessory peptides may be derived from the following proteins and include the indicated amino acid sequences:

Peptide Source	Sequence
EGF receptor	DVVDADEYLIPQ
EGF fragment	CMHIESLDSYTC
Kemptide	RGYALG or RGYSLG
Glycogen synthetase	PLSRTLSVAA
Transferrin receptor	FSLAR
H1 histone	ASGSFKL
Casein kinase II substrate	AAAAAASEEE or AAAAAASDDD
Insulin receptor auto-phosphorylation substrate	DIYETDYYR

Other accessory peptides may be advantageously connected to hydrophobic, membrane-soluble anchoring peptides. These accessory peptides (or accessory compounds) may bind to, or be the substrate of, a cellular cytoplasmic protein or enzyme. Preferred accessory peptides in this regard include the following:

Compound	Structure	Reaction
Aspartate protease inhibitor	VLPFFVL (both D-leu)	Bind protease
Vitamin K-dependent carboxylating enzyme substrate	FALEEI, FALEEL or FALEEV	Carboxylation of glutamates to form gamma-carboxy glutamate
Glycerol		Phosphorylation
Pyridoxal		Phosphorylation
Tetrahydrofolate		Bind folate-requiring enzymes
Pantothenic acid		Bind co-enzyme A synthesizing enzymes
Thiamine		Bind synthetic enzymes for thiamine pyrophosphate

Organic membrane intercalators

The fourth class of enhancing moiety that may be used to enhance retention of a targeting protein conjugate at cell surfaces includes organic membrane intercalators. Organic membrane intercalators include compounds having high lipophilicity, such as fatty acids and fatty acid analogs, bile acids, membrane anesthetics, phospholipids and glycolipids. This class of enhancing moiety provides improved cell surface retention that is particularly desirable, for instance, with radioisotopic-targeting protein conjugates.

Long-chain fatty acids may be attached to targeting protein conjugates by first modifying fatty acid carboxyl groups to form active esters. The active ester form of the fatty acid may subsequently be conjugated to targeting protein lysines or sulfhydryl groups.

If trans-unsaturated fatty acids are used, it is preferred that the double bond be situated near the middle of the fatty acid molecule. Exemplary trans-unsaturated fatty acids in this regard include trans-vaccenic acid and elaidic acid. Long-chain hydrocarbons that may be hydrolyzed to produce carboxylates, phosphonates or phosphates are also preferred organic membrane intercalators within the present invention.

As an example, the lipophilicity of an artibody Fab-conjugate, which can readily diffuse into tumors, may be modulated through the covalent attachment of long-chain fatty acids or fatty acid analogs. The degree of lipophilicity of the fatty acid-targeting protein conjugate (CLC) may be modified by altering the degree of derivitization of the targeting protein conjugate or the chain length of the attached fatty acid. For intravenous administration, CLCs containing fatty acid-targeting protein conjugate preferably remain soluble in aqueous buffer. Alternatively, fatty acid-containing CLCs may require the presence of low levels of detergent to maintain solubility consistent with pharmaceutical administration.

Long-chain fatty acids are readily metabolizable and thus, for some therapeutic applications, it may be preferable to use a non-metabolizable fatty acid analog for covalent attachment to a targeting protein conjugate. A preferred non-metabolizable fatty acid analog in this regard is myristic acid.

Bile salts may also promote transmembrane movement. More particularly, hydrophobic bile salts, such as ursodeoxycholic acid and chenodeoxycholic acid, may be used to facilitate drug absorption (G. S. Gordon et al., *Proc. Natl. Acad. Sci.* 82: 7419–23, 1985). Other suitable organic membrane intercalators within the present invention include fusidic acid and medium chain glycerides (K. Higaki et al., *Pharm. Res.* 5: 309–12, 1988). Medium chain glycerides may be conjugated to a targeting protein through succinylation of a free hydroxyl.

Membrane anesthetics (such as lidocaine and its analogs) and phospholipids (such as phosphatidyl inositol and its analogs) are also preferred organic membrane intercalators within the claimed invention.

In one embodiment of the claimed invention, a fatty acid-targeting protein conjugate CLC first binds to its binding site at the plasma membrane. The initial targeting protein-binding site interaction is followed by a secondary interaction of one or more attached fatty acid side chains with membrane lipids. In the case of a monovalent Fab fragment targeting protein, fatty acid modification may produce an Fab fragment CLC component that has increased binding affinity and prolonged retention on the plasma membrane of the target cell.

Fusion proteins

A DNA sequence corresponding to one or more enhancing moieties selected from the four classes discussed above may be fused to another DNA sequence (corresponding to a targeting protein, a cytotoxic agent and/or an enhancing moiety) to form a fusion protein. Exemplary fusion proteins of the present invention may incorporate: (1) a targeting protein (or portion thereof) and a translocating or anchoring peptide; or (2) the enzymatically active portion of a holotoxin molecule fused to a translocating peptide and an anchoring peptide. In the latter case, the fused protein (for instance, an A chain-translocating peptide-anchoring peptide fusion

protein) may be covalently linked to a targeting protein by a variety of methods, as described previously, in order to form a covalently-linked complex of the claimed invention.

More specifically, a recombinant DNA fusion sequence represented by "toxin-spacer-translocating peptide-spacer-anchoring peptide" may be cloned and expressed according to standard procedures. Briefly, the recombinant DNA fusion sequence is inserted in vitro into an expression vector capable of replication in a particular host microorganism. Typically, the expression vector is derived from a plasmid or a virus. See Old and Primrose, *Principals of Gene Manipulation*, 2d ed., University of California Press, 1981, pp. 104-17; PCT Patent Application Publication No. WO 86/00528; U.S. Pat. Nos. 4,599,311 and 4,704,362; and British Patent No. GB 2,119,804.

An expression vector within the present invention contains "expression signals," i.e., DNA sequences, such as promoters or operators, that are required for transcription of fusion DNA sequences into messenger RNA, which is then translated into the fusion protein. The expression signals must be matched (compatible) with the intended host cell. In addition, the fusion ENA sequence is operably linked to these expression signals by appropriate insertion of fusion protein DNA into the expression vector (i.e., the first codon of the fusion DNA sequence is in the same reading frame as an initiation codon).

A number of expression vector/host cell systems have been developed in the art, and include expression vectors suitable for transforming *Escherichia coli* (Old and Primrose, supra, pp. 32-35 and 46-47), *Bacillus subtilis* (Old and Primrose, pp. 51-53), or yeast (Old and Primrose, pp. 62-68). In addition, "shuttle vectors," which are expression vectors that may be transferred between different host microorganisms, have been described by Storms et al., *J. Bacteriol.* 140: 73-82, 1979; and Blanc et al., *Molec. Gen. Genet.* 176: 335-42, 1979. For instance, shuttle vectors with the capacity to replicate in both *E. coli* and *B. subtilis* are known (Old and Primrose, p. 53). Vectors derived from bacteriophages, such as M13 phage, have also proven useful for cloning foreign genes (Old and Primrose, Chapter 5). Standard procedures may be used to insert a recombinant DNA fusion sequence into a suitable expression vector (e.g., homopolymeric tailing, blunt-end ligation, or by linker molecules) (Old and Primrose, p. 92).

Many suitable methods are known for inserting the recombinant DNA fusion sequence/expression vector into a microbial host, in order to generate a recombinant microorganism which expresses the desired recombinant fusion polypeptide. Microorganisms which are suitable as host cells within the present invention include, but are not limited to, prokaryotes, such as gram-negative and gram-positive bacteria, and eukaryotes, such as yeast or mammalian cell lines. Preferred host cells in this regard include *Escherichia coli* and *Saccharomyces cerevisiae*.

Upon transformation of appropriate recipient host cells with a recombinant fusion protein-expression vector, transformants are screened using conventional procedures. Transformant screening techniques will vary according to the particular gene and vector/host system employed.

Selected transformed host cells are cultured in a suitable growth medium under conditions conducive to the production of the desired fusion protein. If the fusion protein is secreted by the host cell, it may be isolated from the culture medium by conventional protein purification techniques. If the desired fusion protein is intracellular, the transformed, cultured cells are collected and then lysed through either mechanical methods (e.g., sonication, homogenization, freeze-thawing, nitrogen compression-decompression, etc.); chemical methods (e.g., treatment with detergents such as sodium dodecyl sulfate, sulfate, guanidine HCl or NP-40); or enzymatic methods (i.e., lysozyme) or combinations thereof. The desired fusion protein may then be purified from the cellular lysate.

In instances where the fusion protein does not incorporate a targeting protein component, the fusion protein will be chemically linked to a targeting protein, according to methods previously discussed.

Preferred fusion proteins within the present invention include: (1) targeting protein: enhancing moiety fusion proteins; (2) targeting protein: drug carrier: enhancing moiety fusion proteins; (3) targeting protein: toxin enzymatic domain: enhancing moiety fusion proteins; (4) toxin enzymatic domain: enhancing moiety fusion proteins; (5) translocating-anchoring peptide fusion proteins; and (6) enhancing moiety-enhancing moiety fusion proteins.

In summary, formation of a covalently-linked complex (i.e., one or more enhancing moieties covalently attached to a targeting protein conjugate) allows increased retention of the targeting protein conjugate component of the complex at the plasma membrane of a target cell. Attaching combinations of enhancing moieties to a targeting protein conjugate may further enhance membrane retention, rapid and efficient internalization of the CLC, and/or translocation across endosomic membranes into the target cell cytoplasm.

To summarize the examples that follow, Example I discloses preparation of a translocating peptidetargeting protein conjugate CLC. Example II describes preparation of a radionuclide-targeting protein-anchoring peptide CLC. Preparation of an organic membrane intercalator-targeting protein conjugate CLC is presented in Example III: preparation of a fusion protein (toxinenhancing moiety)-targeting protein CLC is disclosed in Example IV. Example V describes preparation of an accessory peptide-enhancing moiety-targeting protein conjugate CLC. Example VI presents in vitro and in vivo assessment of retention and translocation of CLCs.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLE I

Preparation of a Translocating Peptide-Targeting Protein Conjugate CLC

The translocating peptide KGEAALA(EALA)$_4$EALEALAA-amide is synthesized using tea-bag methodology and solid phase peptide synthesis procedures described by Merrifield et al. (*Biochemistry* 21: 5020-31, 1982) and Houghten (*Proc. Natl. Acad. Sci. (USA)* 82: 5131-35, 1985) or using a commercially available automated synthesizer, such as the Applied Biosystems 430 A peptide synthesizer. The peptide-amide is deprotected in 45% trifluoroacetic acid-51% methylene chloride-2% ethanedithiol-2% anisole for 20 min, and cleaved from the 4-methylbenzhydrylamine resin using the Tam-Merrifield low-high HF procedure (J. P. Tam et al., *J. Am. Chem. Soc.* 105: 6442-55, 1983). The peptide is then extracted from the resin using 0.1 M ammo-

nium acetate buffer, pH 8, and is lyophilized. The crude translocating peptide is purified using reverse phase HPLC on a Vydac C-4 analytical column (The Separations Group, Hesperia, Calif.), and a linear gradient of 0.5–1.0%/min from 100% acetonitrile + 0.1% v/v trifluoroacetate to 100% acetonitrile + 0.1% trifluoroacetate. The HPLC-purified peptide is analyzed by amino acid analysis (R. L. Heinriksen and S. C. Meredith, *Anal. Biochem.* 160: 65–74, 1984) after gas phase hydrolysis (N. M. Meltzer et al., *Anal. Biochem.* 160: 356–61, 1987). The sequence of the purified translocating peptide may be confirmed by Edman degradation on a commercially available sequencer (R. M. Hewick et al., *J. Biol. Chem.* 15: 7990–8005, 1981).

The purified translocating peptide is conjugated to a heterobifunctional crosslinking reagent, such as succinimidyl 4-(N-maleimido-methyl)cyclohexane-1-carboxylate (SMCC) through its amino terminus. Briefly, the peptide is dissolved in 0.1 M borate buffer, pH 7–9, and the crosslinker, which is dissolved in buffer with as much DMSO as necessary for solubility, is added in equimolar amounts. The peptide-SMCC mixture is reacted for approximately 30 min at room temperature, and the derivatized product is separated using PD-10 gel filtration. The SMCC-derivatized translocating peptide is then combined at a 5:1 ratio with an A chain cytotoxic agent (such as ricin A chain) that has been prereduced with dithiothreitol (DTT) and separated from B chain by reactive blue 2 {(1-amino-4[[4-[[4-chloro-6-[[3(or4)-sulfophenyl]amino]-1,3,5-triazin-2-yl]amino]-3-sulfophenyl]amino]-9,10-dihydro-9,10-dioxo-2-anthracenesulfonic acid)}-sepharose chromatography. The reduced ricin A chain reacts with the maleimide group of the SMCC-derivatized peptide, forming a thioether bond; unreacted derivitized peptide is quickly removed by gel filtration.

The translocating peptide-modified ricin A chain is reacted with iminothiolane to generate further thiol groups, which are then used to create a disulfide bond with DTT (50mM)-treated antibody. The translocating peptide-ricin A chain-antibody CLC is separated from unreacted ricin A chain-translocating peptide by gel filtration on an HPLC TSK 3000 column (BioRad, Richmond, Calif.) using a flow rate of 0.5 ml/min in phosphate-buffered saline (PBS), 0.1 M, pH 7.2.

EXAMPLE II

Preparation of a Radionuclide-Targeting Protein-Anchoring Peptide CLC

A. Chelation and Radiolabeling of a Targeting Protein Prior to Peptide Conjugation

Anchoring peptide AVGAIGAMFLGF-LGAAGSTMGAASL is chemically synthesized according to the methodology described in Example I. Purified anchoring peptide is conjugated to sulfo-SMCC, a water-soluble heterobifunctional crosslinking reagent, using a modification of the procedure described in Example I (reducing the organic solvent).

Monoclonal antibody NR-CO-02 is prelabeled with ^{188}Re-MAG$_2$-GABA. Briefly, $^{188}ReO_4^-$ tetrabutylammonium+, obtained from a $^{188}W/^{188}Re$ generator, is loaded onto a C_{18} reverse phase cartridge (Ultrasphere 5 micron, 1.5 ml/min flow), washed with water to remove nitrates, dried and eluted with ethanol onto strong cation exchange cartridges equilibrated with Li^+. The Li^+ salt of ReO_4^- elutes directly and is dried under N_2 at 75° C. A stannous citrate solution is added, followed by the addition of 2,3,5,6-tetrafluorophenyl S-ethoxyethylthioacetylglycyl glycyl-gamma-aminobutyrate (MAG$_2$-GABA) dissolved in isopropanol. The reaction mixture is incubated at 75° C. for 30 min, with formation of a ^{188}Re-N$_2$S$_2$-TFP active ester complex. The ^{188}ReN$_2$S$_2$-TFP active ester complex is purified on cartridges of the cation exchange resin, then on C_{18} reverse phase cartridges. More specifically, the ester is loaded, washed with water and water-ethanol, dried under low vacuum, and eluted with ethanol. The resultant solution is dried under blowing N_2 at 75° C., and monoclonal antibody in 0.2 M sodium cartonate buffer, pH 9.5, is added. After 15 min, the labeling reaction is terminated by the addition of lysine, which reacts with unreacted active ester. The monoclonal antibody-^{188}Re-MAG$_2$-GABA conjugate is purified by gel filtration prior to attachment of an anchoring peptide or anchoring peptide-spacer peptide.

Sulfo-SMCC-derivatized anchoring peptide is reacted with free sulfhydryl groups generated on the ^{188}Re-MAG$_2$-GABA-monoclonal antibody by reduction with 50 mM DTT. Unbound derivatized anchoring peptide is removed by gel filtration.

Alternatively, a monoclonal antibody-anchoring peptide conjugate may be post-labeled with ^{188}Re. Briefly, monoclonal antibody is reacted with unlabeled N$_2$S$_2$-TFP active ester, in a manner analogous to the procedure described for prelabeling with ^{188}Re-MAG$_2$-GABA. The monoclonal antibody-MAG$_2$-GABA molecule is conjugated with an enhancing moiety, as detailed above, and the monoclonal antibody-MAG$_2$-GABA-enhancing moiety conjugate is then post-labeled with ^{188}Re.

B. Peptide Conjugation to a Targeting Protein Prior to Chelation for Radiolabeling

If an anchoring peptide and/or peptide-spacer are non-reactive with a chelating agent to be used for radiolabeling, the peptide or spacer moiety may be conjugated to a targeting protein prior to attachment of the chelator.

For instance, succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB) is conjugated to a purified anchoring peptide lacking lysine residues, according to the procedure described in Example I. The SMPB-derivatized anchoring peptide is then reacted with free sulfhydryl groups provided on melanocyte stimulating hormone according to the procedure of Example II.A.

The anchoring peptide-targeting protein conjugate is then reacted with ^{188}Re-N$_2$S$_2$-TFP active ester complex, as described in Example II.A. The melanocyte stimulating hormone-anchoring peptide-^{188}Re-MAG-GABA complex may be purified prior to in vivo administration.

EXAMPLE III

Preparation of an Organic Membrane Intercalator-Targeting Protein Conjugate CLC

A. Synthesis of Active Ester of Myristic Acid

An active ester form of myristic acid is obtained through a dicyclohexylcarbodiimide (DCC)-mediated condensation of myristic acid and sulfo-N-hydroxysuccinimide (sulfo-NHS) in a dimethyl-formamide (DMF) solution. To 0.44 ml of DMF is added 0.05 g (0.22 mmole) myristic acid, followed by 0.052 g (0.24 mmole) sulfo-NHS and 0.05 g (0.24 mmole) DCC. Upon addition of DCC, the reaction solution becomes cloudy with

the formation of dicyclohexylurea. The reaction mixture is stirred at room temperature for 1 h, and then at −20° C. for 2 h, The reaction mixture is filtered and the solid discarded. The solvent is removed from the filtrate, and the resulting viscous residue is overlaid with methylene chloride. This mixture is stirred overnight at room temperature, and the resultart solid is filtered and dried to obtain the desired active ester of myristic acid (0.09 g) in 97% yield. The active ester product is characterized by thin layer chromatography and nuclear magnetic resonance.

Myristic acid active ester is then conjugated to an antibody $F(ab')_2$ fragment using a 10:1 offering ratio, to provide an average of 1-3 fatty acid molecules per protein conjugate. An active ester form of verrucarin A is then reacted with the antibody $F(ab')_2$ fragment, either before or after chemical linkage of the fatty acid.

EXAMPLE IV

Preparation of a Fusion Protein (Toxin-Enhancing Moiety) - Targeting Protein CLC

A ricin A chain-translocating peptide-anchoring peptide fusion protein is produced through recombinant DNA technology. Briefly, the C-terminus of a DNA sequence encoding ricin A chain is ligated by conventional procedures (e.g., using T_4 DNA ligase) to a DNA sequence corresponding to a GGG spacer. The C-terminus of the ricin A-GGG DNA sequence is then fused to the N-terminus of a DNA sequence encoding the translocating peptide KGEAALA(EALA)-4EALEALAA.

The N-terminus of a DNA sequence encoding the anchoring peptide AVGAIGAMFLGFLGAAGST-MGAASLC-cys is ligated to a DNA sequence corresponding to a GGG spacer; the N-terminus of the GGG spacer-anchoring peptide-cys DNA sequence is then ligated to the C-terminus of the ricin A-GGG spacer-translocating peptide DNA sequence. The resultant fusion product is diagrammed below.

Ricin A chain—GGG—KGEAALA(EALA)4EALEALAA—...
(toxin; translocating)

...—GGG—AVGAIGAMFLGFLGAAGSTMGAASL—C
(anchoring)

Alternatively, peptide-spacer DNA sequences may be synthesized in vitro using standard oligonucleotide synthesis procedures (see, e.g., U.S. Pat. Nos. 4,500,707 and 4,668,777).

The recombinant ricin A-translocating peptide-anchoring peptide-cys DNA sequence is cloned in an *E. coli* expression vector using conventional procedures. *E. coli* strain HB101 is transformed with the fused recombinant DNA sequence and cultured to produce the ricin A-translocating peptide-anchoring peptide-cys fusion protein. The fusion protein is purified from the transformed *E. coli* culture by standard methods, such as anti-ricin A affinity chromatography or reactive blue 2-sepharose chromatography. The fusion protein may be eluted from the affinity matrix using standard techniques, such as high salt, chaotropic agents, or high or low pH.

The ricin A-translocating peptide-anchoring peptide-cys fusion protein is combined with DTT-treated monoclonal antibody according to the procedure of Example I, in order to obtain a ricir A-translocating peptide-anchoring peptide-monoclonal antibody CLC. The incorporation of both a translocating peptide and an anchoring peptide into the toxin immunoconjugate CLC provides increased cellular membrane interaction, and may provide a corresponding increase in internalization and translocation of the CLC.

EXAMPLE V

Preparation of an Accessory Peptide-Enhancing Moiety-Targeting Protein Conjugate CLC

A translocating peptide having an accessory peptide attached at its C terminus may be chemically constructed in a single synthetic process. Briefly, a "translocating-accessory peptide" enhancing moiety composed of the translocating peptide CGEAALA(EALA)4EALEALAA and the casein kinase II substrate accessory peptide AAAAAASEEE is synthesized according to the procedure in Example I. The resultant translocating-accessory peptide is depicted below.

CGEAALA(EALA)-
4EALEALAAAAAAAASEEE-amide

The translocating-accessory peptide enhancing moiety may be either: (1) directly attached through its N terminal cysteine to free sulfhydryls present on a DTT-treated targeting protein; (2) attached to a targeting protein by means of a heterobifunctional crosslinker, such as SPDP (see Example I); or (3) attached to a targeting protein via a spacer peptide. The translocating-accessory peptide-targeting protein conjugate is then covalently linked to a trichothecene according to methodology described in U.S. Pat. No. 4,744,981.

Upon in vivo administration of the translocating-accessory peptide-targeting protein-trichothecene CLC, the targeting protein component binds to an appropriate binding site on a target cell. After initial binding and internalization of the targeting protein component, the translocating peptide component of the conjugate traverses the target cell endosomic membrane, causing the accessory peptide component to protrude into the cytoplasm of the target cell. The C terminal 10-mer of the accessory peptide serves as a substrate for the intracellular enzyme casein kinase II, and the serine residue of the 10-mer becomes available for phosphorylation.

Another synthetic translocating-accessory peptide is represented by the following amino acid sequence:

CGEAALA(EALA)4EALEALAADV-
VDADEYLIPQ-amide

The C terminus of the accessory peptide portion of this synthetic peptide serves as a substrate for tyrosine kinase.

Yet another synthetic translocating-accessory peptide contains a spacer region CDNDNDDNDDGGG at the N terminus. A synthetic peptide having an N terminal spacer is illustrated below.

CDNDNDDNDDGGGCGEAALA(EALA)-
4EALEALAAFSLAR-amide

Synthetic peptides of this length may be obtained using an Applied Biosystems 430 A peptide synthesizer, following the manufacturer's N-methylpyrrolidone-DMSO coupling procedure. Alternatively, a spacer-translocating-accessory peptide enhancing moiety may be synthesized using manual solid phase methcdology,

as described in Example I. With manual solid phase synthesis, it is preferred that the coupling of all amino acid residues after amino acid 20 is quantitatively monitored by ninhydrin methodology (V. Sarin et al., *Anal. Biochem.* 117: 147-57, 1981). If coupling is less than 99.0% complete at any step, the suboptimally coupled amino acid preferably is coupled a second time, or until coupling is greater than 99.0% complete.

Because longer synthetic peptides may be somewhat heterogeneous, additional purification beyond reverse phase HPLC chromatography, as described in Example I, may be required. For instance, HPLC-ion exchange protocols (F. Regnier, *Meth. Enzymol.* 91: 137, 1983) or hydrophobic interaction chromatography may be used for further purification of heterogeneous synthetic peptides.

An anchoring-accessory peptide enhancing moiety may be synthesized according to the following scheme:

tyr-accessorypeptide-GGG-anchoringpeptide-GGG-cys

The N terminal tyrosine serves as a substrate for tyrosine kinase of H-ras, which is present on the cytoplasmic side of plasma membranes of transformed cells.

A translocating-accessory peptide or an anchoringaccessory peptide enhancing moiety may be conjugated to an targeting protein conjugate according to the procedures described in Examples I and II.

EXAMPLE VI

Assessment of Enhanced Cellular Retention and Translocation of CLCs

Covalently-linked complexes according to Example II or Example IV are assayed for cellular retention by radiolabeling the CLC according to the procedure detailed in Example II, or by any standard radiolabeling methods known in the art. Aliquots of CLC (2-6 ng of targeting protein) are added to 1×10^6 binding site-positive target cells in 200 μl Dulbecco's minimal essential medium (DMEM) containing 5% fetal bovine serum (FBS). The CLC-target cell mixture is incubated for 1-2 h at 4° C. Following two washes with DMEM, the target cells are resuspended in 200 μl of DMEM-FBS medium and incubated for various periods of time (up to 24 h) at 37° C. Replicate samples are removed at specified time periods and assayed for cell-associated radiolabeled CLC. The assay procedure involves layering the removed cell samples over 1 ml dibutyl- or dinitrophthalate oils, followed by centrifugation at 200×g for 10 min. The centrifuge tubes are cut in half, and the radioactivity associated with the cell pellet is determined. CLCs will demonstrate enhanced retention of cell-associated radioactivity over time as compared to the corresponding unmodified conjugate.

Enhanced in vivo retention of CLCs is determined using nude mouse xenografts of human tumor tissue. A radiolabeled CLC according to Example II or IV that is directed against colon tumor cells is administered intravenously to nude mice xenografted with human colon tumor cells (LS-180). The mice are sacrificed at a various times post-administration, and organs are removed and assayed for radioactivity. CLCs will show prolonged retention in tumor tissue as compared to unmodified conjugates.

Enhanced translocation of toxin-containing CLCs (Examples I and V) is assayed by the following procedure. Toxin activity is determined by means of cytotoxicity tests using the mitochondrial dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT; Sigma) (T. Mosmann, *J. Immunol. Meth.* 65: 55 (1983)), or by measuring inhibition of ^{3}H-leucine incorporation into protein. Toxin CLCs will demonstrate increased potency as compared to unmodified toxin conjugates.

Alternatively, toxin-CLCs are radiolabeled according to standard procedures, and cell-associated/internalized radioactivity determined as described above. By this procedure, the amount of radiolabel on the cell surface or within target cells may be determined. Briefly, aliquots of cells having bound radiolabeled conjugates are incubated with trypsin in sufficient amounts to remove cell surface antigen-antibody complexes. Any remaining cell-associated radiolabel (insensitive to trypsin) has been internalized. Toxin- or drug-CLCs will demonstrate an increase in internalized radiolabel as compared to corresponding radiolabeled unmodified conjugates.

As a direct measure of translocation, conjugates are tested in a translocation assay for pH-dependent membrane binding. For comparison of an unmodified ribosomal inactivating protein (RIP) and a translocating peptide-RIP, samples are incubated in test tubes containing an appropriate volume of 1 mM glycine buffer, pH 5.0 for 30 min at 37° C. These conditions mimic the environment within a target cell endosome. Target cells are resuspended in the same buffer, added to the toxin or translocating peptide-toxin samples, and incubated at 37° C. for 5 min. The cell suspensions are brought to neutrality with 0.1 M Tris, pH 8.0, and cytotoxicity is measured following a further incubation of 48 h. Positive controls include diphtheria toxin, which is known to possess pH-dependent translocating activity, and incubation at pH 7.0 rather than pH 5.0. In a similar manner, targeting protein conjugates containing toxins or modified toxins may be assayed.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

What is claimed is:

1. A method for enhancing in vivo cytotoxicity of a targeting protein conjugate comprising administering to a tumor-bearing patient a therapeutically effective amount of a covalently-linked complex (CLC) comprising a targeting protein or a targeting peptide; a cytotoxic agent; and an enhancing moiety capable of promoting CLC-membrane interaction, wherein the enhancing moiety exhibits alpha helical structure at acidic pH and substantially unfolds at physiological pH and exhibits little or no tertiary structure.

2. The method of claim 1 wherein the targeting protein or targeting peptide is an antibody, an antibody fragment or an antigen-binding portion of an antibody.

3. The method of claim 1 wherein the cytotoxic agent is a radionuclide; a toxin or a fragment or analog thereof; a drug or an analog thereof; or a combination of the foregoing.

4. The method of claim 3 wherein the radionuclide is selected from the group consisting of ^{188}Re, ^{186}Re, ^{203}Pb, ^{212}Pb, ^{67}Cu, ^{90}Y, ^{125}I, ^{131}I, ^{77}Br, ^{211}At, ^{97}Ru, ^{105}Rh, ^{198}Au, ^{199}Au and ^{119} Sb.

5. The method of claim 1 wherein the enhancing moiety is a translocating peptide, an anchoring peptide, an organic membrane intercalator or a combination thereof.

6. A method of claim 1 wherein the targeting protein or targeting peptide comprises a synthetic protein or synthetic peptide.

7. A method of claim 1 wherein the targeting protein or targeting peptide comprises an analog of an antibody, an antibody fragment or an antigen-binding portion of an anitbody that retains the capacity to bind to a defined target cell population.

8. A method for enhanced in vivo imaging of a tumor comprising administering to a tumor-bearing patient a diagnostically effective amount of a covalently-linked complex (CLC) comprising a targeting protein or a targeting peptide; a radionuclide selected from the group consisting of gamma-emitters, positron emitters and X-ray emitters; and an enhancing moiety capable of promoting CLC-membrane interaction, wherein the enhancing moiety exhibits alpha helical structure at acidic pH and substantially unfolds at physiological pH and exhibits littel or no tertiary structure.

9. The method of claim 8 wherein the targeting protein or targeting peptide is an antibody, an antibody fragment or an antigen-binding portion of an antibody.

10. The method of claim 8 wherein the radionuclide is selected from the group consisting of ^{188}Re, ^{186}Re, ^{67}Cu, ^{131}I, ^{97}Ru, ^{105}Rh, ^{123}I, ^{111}In, ^{67}Ga, ^{99m}Tc and ^{18}F.

11. The method of claim 8 wherein the enhancing moiety is a translocating peptide, an anchoring peptide, an organic membrane intercalator or a combination thereof.

12. A method of claim 8 wherein the targeting protein or targeting peptide comprises a synthetic protein or a synthetic peptide.

13. A method of claim 8 wherein the targeting protein or targeting peptide comprises an analog of an antibody, an antibody fragment or an antigen-binding portion of an antibody that retains the capacity to bind to a defined target all population.

* * * * *

EXHIBIT 6

Scintigraphic Detection of Metastatic Melanoma Using Indium 111/DTPA Conjugated Anti-gp240 Antibody (ZME-018)

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We evaluated the toxicity, pharmacokinetics, and localization of a monoclonal IgG_{2a} murine anti-human melanoma (gp240) antibody (ZME-018) that recognizes a tumor-associated cell surface glycoprotein of 240,000 molecular weight present in most melanomas. The antibody was conjugated with DTPA (diethylenetriamine pentaacetic acid) and labeled by chelation of ^{111}In. One mg of antibody labeled with 5 mCi of ^{111}In was infused, together with 0 to 40 mg of "cold" carrier ZME-018. The blood clearance, urinary excretion, and in vivo localization were determined in 26 patients. Scintigraphic images were obtained at 24 hours and 72 hours in all patients. Mild toxicity occurred in one patient. The half-time clearance of labeled monoclonal murine antibody (MoAb) from the blood increased from 16.1 hours at an antibody dose of 1 mg to 35.9 hours at 40 mg. Males showed faster clearance from the blood than did females or a single castrated male, perhaps due to selective concentration of antibody in the testes. Nonspecific uptake in liver, spleen, bone marrow, and intestine was seen in all patients. The percentage of known metastatic foci detected increased with the total dosage of antibody, from 23% at doses ≤ 5 mg, to 65%, 87%, and 78% for 10, 20, and 40 mg, respectively. We conclude that at doses of ≥ 10 mg, ZME-018 is a safe and potentially useful agent for the scintigraphic detection of metastatic malignant melanoma.

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MELANOMA is a disease of increasing incidence for which no therapeutic intervention after primary surgery has a proven survival advantage.[1,2] The optimal use of existing palliative treatments, as well as the application of alternative new investigational strategies, rests heavily upon the early detection of metastatic disease. When metastatic melanoma is at its smallest, it is theoretically most susceptible to immunological, chemotherapeutic, and other systemic interventions.[3] Diagnostic scintigraphic techniques for the detection of metastatic melanoma offer dynamic advantages over purely anatomic, static radiographic techniques, but no highly sensitive and specific means for scintigraphic detection of metastatic melanoma has been developed.[4] A variety of murine monoclonal antibodies (MoAb) against cell-surface and other antigens of melanoma have been developed that detect antigens of different molecular sizes and degrees of restriction to melanoma. Multiple laboratories have thus identified immunologically functional Ia antigens in melanoma,[5-7] and other tumor antigens that appear to represent markers of tissue differentiation.[8,9] Other antigens are markers of potential physiological significance in neoplasia and fetal tissues.[10,11] Gp240 is a melanoma-associated antigen that has exhibited greater restriction to melanoma than other antigens.[12-17] Gp240 has been found in 80% to 94% of melanoma specimens studied in a comparative examination of multiple MoAbs tested against a range of neoplastic and nonneoplastic melanocytic lesions in our laboratory.[18] Immunohistological studies of melanoma and other melanocytic processes with several murine MoAbs, including the antibody ZME-018, have shown the presence of gp240 antigen more frequently than p97 antigen[10] and have prompted our pursuit of this antibody to gp240 for immunoscintigraphic detection of metastatic melanoma.[19]

Here we report phase I-II radioimmunoscintigraphic studies of ZME-018 in which we have

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found no significant toxicity, and preliminary encouraging data regarding the diagnostic capabilities of this antibody administered in escalating doses to 26 subjects with metastatic melanoma. The antibody was covalently coupled to the bifunctional chelator, diethylenetriamine pentaacetic acid (DTPA), and labeled by chelation of ^{111}In for this study. This radiometal avoids the problem of dehalogenation that has occurred with radioiodinated antibodies; and has been reported to yield improved tumor-blood ratios of radioactivity in comparison to radioiodinated antibody.[20-22] Others have reported comparable pharmacokinetics of plasma ^{111}In radioactivity and antibody clearance (determined by enzyme-linked immunoabsorbent assay), with the 96.5 antibody to the melanoma p97 antigen labeled by a similar chelation technique. Unfortunately, this IgG_{2a} antibody to p97 was capable of detecting only approximately 50% of 100 known tumor sites.[21]

Overall, there is clearly a need for the exploration of new imaging methods for the detection of metastatic melanoma, and monoclonal antimelanoma antibodies provide the most refined tools available to achieve this.

MATERIALS AND METHODS

Preparation of ZME-018 MoAb Labeled With ^{111}In

The specificity and distribution of the melanoma-associated antigen gp240 as detected by ZME-018 has been described.[15-17,23] The antibody is an IgG_{2a} murine monoclonal hybridoma product harvested from Balb/c mouse ascites and purified by diethylaminoethyl (DEAE) column chromatography. The antibody was conjugated with the chelating agent, DTPA, using a modification of the method of Krejcarek and Tucker.[24] It was provided in a sterile and nonpyrogenic form, with normal human serum albumin as carrier protein, for radionuclide chelation immediately before clinical use. A uniform dose of 5 mCi ^{111}In was used to avoid the problems reported by others with the use of varying quantities of radionuclide.[21] Antibody reactivity to known gp240-positive cultured melanoma lines (Y Mel 84-420 and Y Mel 81-180) was determined to assure preservation of radiolabeled antibody immunoreactivity in representative DTPA-conjugated ^{111}In chelated antibody preparations. These tests revealed a minimum of 29% to 32% antibody uptake with 6.2 μg antibody in 0.5 mL medium incubated 24 hours at 4°C over 2.0×10^6 melanoma cells. No binding to gp240-negative fibroblast cultures was observed.

Antibody ZME-018, conjugated with DTPA, was provided by Hybritech, Inc, San Diego, in sterile pyrogen-free aliquots (1 mg each). The conjugated antibody was radiolabeled with a buffered solution of ^{111}In. Instant thin-layer chromatography was used as a test to confirm the association of the radioactivity with the MoAb. The labeling efficiency was 94.9% ± 1.56%.

Subjects

Twenty-six subjects with histologically confirmed malignant melanoma and measurable tumor documented by physical examination, or radiographic studies, gave written informed consent to this study. The study protocol was approved by the Yale Human Investigations Committee. Seventeen men and nine women of median age 55 (range, 25 to 87) were enrolled. Table 1 summarizes the demography of the patients, sites of known disease, and quantities of antibody administered. Patients no. 5, 6, and 9 entered this study having had metastatic disease in regional lymph nodes in the past, and each had developed palpable or radiographic lesions suspected of being further recurrence of melanoma. However, subsequent surgical exploration or imaging studies in each failed to confirm active tumor that could have been imaged at the time of antibody study. In addition, patient no. 24 had a single skin metastasis 3 mm in diameter, and did not show evidence of any other metastases on antibody scintigraphy or other restaging studies. Thus, these four patients contributed only to the toxicity and pharmacokinetics analysis of the study.

Patients gave negative histories for prior murine antibody exposure, and had negative intradermal skin test reactions to the antibody solution (1 μg MoAb). Complete medical oncology history, physical examination, EKG, and hematologic/urinary/blood chemistry profiles were obtained in all patients. Documentation of metastatic disease included the enumeration of all disease sites by physical examinations, PA and lateral chest radiographs, tomographic gallium 67 scans, ^{99m}Tc sulfur colloid liver-spleen scans, abdominal ultrasound studies, and computed tomography (CT) brain and body scans. The gallium scans were obtained within 4 weeks of antibody imaging in 13 of the study patients, and within 12 weeks in 17 of the study patients. Where applicable, CT scans were obtained within 4 to 12 weeks as well. The size of metastases was determined by physical examination and on CT scans or radiographs; CT scans were best for this purpose.

Study Methods

Study patients were observed throughout the administration of antibody and for four hours thereafter in the outpatient medical oncology clinic. The radiolabeled 1 mg of MoAb was mixed with a variable quantity of "cold" MoAb, and infused intravenously (IV) in 100 mL 0.9% NaCl solution over one hour. Groups of two to five patients entered each dosage tier as shown in Table 1. Each patient received the MoAb only once. Multiple images of patients were obtained at 24 and 72 hours, and at optional later times, using a gamma camera with computerized image storage (General Electric 400AT and Star Systems, Bridgeport, CT). Patients were clinically examined at a minimum frequency of monthly, with CT and ^{67}Ga performed in most within a month of antibody imaging. Antibody images were scored in a multidisciplinary conference, including medical oncology, radiology, nuclear medicine, and nursing/data management personnel. Sites of uptake were identified by nuclear medicine and scored only if concurred upon by all participants in the multidisciplinary effort.

Table 1. Characteristics of Study Patients and Disease Sites Detected Anti-gp240 by Antibody Imaging

Patient No.	Sex/Age	Ab Dose (mg)	Disease Sites Detected/Known: Peripheral Nodes	Soft Tissue	Lungs	Mediastinum and Hila	Abd Nodes	Abd Masses	Liver	Bone	Brain	Total	False-Positives
1	M/87	1.0	2/3							0/1		2/4	1-Bone met MM
2	M/33	1.0	1/3	0/7	0/2			0/1				1/13	0
3	M/59	2.5	1/2									1/2	0
4	M/51	2.5						1/1				1/1	0
5	M/34	2.5										0/0	1-Ax
6	F/55	2.5										0/0	2-Ax, heart
7	M/70	5.0	1/1		0/1							1/2	3-Ax, heart; thyroid
8	M/65	5.0			0/1	0/1						0/2	2-Ax, frontal sinusitis
9	F/62	5.0										0/0	4-Ax, breasts, heart, colostomy stoma
10	M/52	5.0			0/5							0/5	3-Ax, thyroid, heart
11	F/69	5.0		1/1								1/1	2-Ax, knee ligaments
12	F/49	10.0	1/1	1/1	0/1		2/2					4/5	3-Ax, breasts, heart
13	F/33	10.0			0/1	1/2						1/3	2-Ax, heart
14	F/66	10.0	2/2		2/5		1/1	2/3	0/1			7/12	1-Ax
15	F/36	10.0			2/3				1/1			3/4	2-Ax, heart
16	M/59	10.0		1/1	1/1							2/2	1-Ax
17	M/56	20.0	1/1		0/3	0/1						1/5	1-Ax
18	F/36	20.0				1/3		1/1				2/4	2-Ax, breasts
19	M/64	20.0	1/2		0/1	0/1						1/4	0
20	M/75	20.0			1/1							1/1	1-Ax, bone (prostate met)
21	F/72	20.0		54/54		1/1						55/55	0
22	M/60	40.0		1/1			0/2	1/1				2/4	2-Ax, bowel
23	M/25	40.0									1/1	1/1	1-Ax
24	M/51	40.0										0/0	1-Ax
25	M/58	40.0							0/1	2/2		2/3	1-Ax
26	M/41	40.0	5/5	9/12		1/1	1/2	3/3	1/1			20/24	0
Total sites by location			15/19	67/77	6/25	4/10	4/7	8/10	2/4	2/3	1/1	109/156	
Percentile detection			79	87	24	40	57	80	50			70	

Abbreviations; Ab, antibody; Abd, abdominal; met, metastases; Ax, axilla.

Table 2. Antibody Clearance According to Dose Infused

Total* Antibody Dose (mg)	Patients (n)	Clearance From Circulation (T½ h ± SD)
1.0	2	16.1 ± 0.5
2.5	4	19.6 ± 1.9
5.0	5	26.4 ± 5.2
10.0	5	27.4 ± 7.2
20.0	5	30.8 ± 12.9
40.0	5	35.9 ± 7.7

Note. Cumulative urinary excretion in 48 hours is 6.1% ± 2.3% of infused dose of radioactivity.
*Including 1.0 mg labeled DTPA-coupled antibody.

Measurement of Blood Clearance and Urinary Excretion of Radioactivity

Whole blood samples (5 mL) were taken at 0 and 30 minutes, and 2, 3, 24, and 72 hours after antibody infusion. Urine collections were obtained from all patients up to 48 hours postinfusion. Duplicate 100 μL blood and urine samples were counted for radioactivity by scintillation spectrophotometry. The counts were adjusted for the physical decay of ^{111}In, and pharmacokinetics calculated by nonlinear regression analysis.

RESULTS

Pharmacokinetics of ZME-018 Anti-p240 Monoclonal Antibody Labeled With ^{111}In

Table 2 shows the whole blood clearance and urinary excretion of radionuclide label according to the dose of antibody infused.

Toxicity

One patient demonstrated mild toxicity. She had a history of allergies and asthma. Her preliminary skin test was negative. Approximately 90 minutes after the infusion was begun, she complained of headache, nasal congestion, and mild wheezing. A mild rash developed. Benadryl (Park-Davis, Morris Plains, NJ) 25 mg, per os, resulted in complete relief.

Radioimmunoscintigraphy Results

The distribution of known tumor sites ≥ 1 cm in diameter is given in Table 1. The disease in this population is representative of the population with metastatic melanoma followed in the Yale Melanoma Unit. It is notable that an increasing fraction of known sites of disease was detected as the total dosage of antibody was increased from 1 mg to 40 mg (Table 3). The overall sensitivity for the entire study was 109 of 157 metastatic foci, or 70%. For the 2.5 mg dose of antibody, only three tumor sites were studied, so the data at this dose tier are unreliable. However, for all antibody dosages ≤ 5 mg, the sensitivity was seven of 30 or 23%. Above this dosage, there was an increase in sensitivity, but this was not significantly different among the 10, 20, and 40 mg doses. The pooled data for these three groups (10, 20, and 40 mg) gave a sensitivity of 102 of 127 or 80% of known sites of 1 cm diameter or greater. In patient no. 21, the large number of lesions and the high sensitivity of detection may bias the sensitivity data for the 20-mg group.

Patients no. 4, 21, and 26 were found to have multiple previously unknown tumor sites in abdomen and soft tissue, which were confirmed by subsequent clinical or imaging examinations. Numerous foci of previously unknown disease in patients no. 21 and 26 were detected at the limit of standard imaging techniques. These are not included in the tables, since they were confirmed by later evolution or by ^{67}Ga studies and were thus of unknown exact size at the time of ZME-018 imaging (See Figs 1 and 2).

Table 3. Summary of Tumor Detected by ZME-018 ^{111}In According to Dosage of Antibody Infused

MoAb Dose (mg)	Patients (n)	Total of Known Tumor* Sites	True-Positive	False-Positive	False-Negative	Overall Sensitivity (%)
1.0	2	17	3	1	14	18
2.5	4	3	2	3	1	67
5.0	5	10	2	14	8	20
10.0	5	26	17	9	9	65
20.0	5	69	60	4	9	87
40.0	5	32	25	5	7	78
						(x = 70)

*Sites of ≥ 1 cm.

The sensitivity of ZME-018 antibody imaging according to the location of the metastases is presented in Table 1. Metastases in soft tissue, such as skin, subcutaneous tissue, and muscle, were most accurately detected (87%). Seventy-nine percent of peripheral lymph node foci of tumor were detected. Lung metastases were poorly seen (25%). The numbers for other individual anatomic areas are smaller and less reliable. However, as data for all abdominal sites are pooled, 14 of 21 or 67% were detected.

The antibody localized nonspecifically in liver, spleen, bone marrow, and intestine of all patients, and to the axillae of a majority. The testes of all noncastrate males demonstrated preferential uptake, without relation to the presence of tumor. The sites of false-positive uptake are listed in Table 2. These were not a source of serious confusion with tumor, except for the colostomy stoma (no. 9) and thyroid (no. 7) uptake. Cardiac uptake occurred in seven patients (no. 6, 7, 9, 10, 12, 13, and 15), and was differentiated from tumor by concurrent and follow-up chest radiographs, CT scans, and ultrasound.

DISCUSSION

The present phase I-II radioimmunoscintigraphy study of the murine MoAb ZME-018, directed against the melanoma-associated glycoprotein gp240, presents the first pharmacokinetic and imaging results with this ^{111}In-DTPA labeled MoAb. Previous in vitro immunohistological studies with anti-gp240 MoAb have compared favorably with parallel studies of the MoAb 96.5 anti-p97, the most extensively studied imaging agent for metastatic melanoma.[10,11,13-16,18,21-23] In our studies, there was a high and consistent radioactive labeling of antibody. There was also acceptable binding of this radiolabeled antibody to a gp240-positive tumor cell line, Y Mel 84-420, indicating preservation of antibody reactivity. Previous major studies of the imaging sensitivity of radiolabeled antibody to melanoma antigens have involved ^{111}In labeling of the 96.5 antibody to the p97 antigen, by means of DTPA chelation.[24,33] One milligram of radiolabeled antibody was mixed with increasing amounts of cold antibody, providing a total MoAb dosage range of 0.5 to 20.0 mg. Overall, 50 of 100 metastatic foci were detected (sensitivity, 50%). In our study, the overall sensitivity was 109 of 157 (69%). In the 96.5 study, sensitivity for an antibody dose $\leq$5 mg was 25%, and in our study 23%. For a total 96.5 antibody dose of 10 and 20 mg, sensitivity was 68%, whereas in our pooled 10-, 20-, and 40-mg groups, sensitivity was 81%. The ^{111}In-96.5 study used only 2.0 to 2.5 mCi of ^{111}In, except for one test site. That group received 5.0 mCi at a total MoAb dose of 20 mg; sensitivity was 81%, whereas in our comparable dosage tier, the sensitivity was 87% (Table 3). Thus, our results confirm that imaging with increased doses of cold antibody may demonstrate a greater proportion of metastatic foci. Our results are also encouraging in comparison to results reported with directly radioiodinated antibodies, and F(ab')$_2$ fragments labeled with ^{99m}Tc, ^{111}In, ^{123}I, or ^{131}I.[25-28]

A ten-center study of ^{111}In and ^{99m}Tc-labeled F(ab')$_2$ fragments of an antibody similar to the high molecular weight antigen of melanoma has been recently published. This work employed no standard corollary imaging techniques for disease confirmation and variably employed "subtraction" methods due to high background uptake of ^{99m}Tc. The authors conclude that F(ab')$_2$ labeled with ^{99m}Tc may be superior to F(ab')$_2$ labeled with ^{111}In, but report detection of 94 of 159 lesions (59%) of various sizes using 5 mCi ^{111}In with 2 mg F(ab')$_2$. While we have detected 109 of 156 lesions (70%) in the study of intact anti-p240 ^{111}In, the differences due to larger quantity of intact antibody here preclude simple comparison with the results of Siccardi et al.[29] It is notable that 2 mg of F(ab')$_2$ labeled with 10 to 30 mCi ^{99m}Tc detected 74% of known tumor masses in that study, whereas the use of anti-p240 labeled with 1 mCi ^{111}In and 10, 20, or 40 mg cold antibody here allowed detection of up to 78% to 87% of tumor sites. A correlation between lower stage of disease and imaging success was not apparent in this study, where lesions imaged were all detected in stage III to IV patients. If the findings of the multicenter trial reported by Siccardi are valid, one may project improved results of the anti-p240 antibody in stage II patients, arguing for earlier use of this diagnostic modality.

This study has demonstrated localization of the anti-p240 antibody in a sufficient number of tumor foci in skin, subcutaneous tissues, muscle, and peripheral lymph nodes to suggest a possible

future clinical utility of this technique for disease detection in these areas. There is a critical need to improve the precision of staging, and specifically, the assessment of regional lymph nodes of patients at high risk of relapse. Immunoscintigraphy with anti-p240 provides an avenue by which regional lymph node status might be assessed preoperatively. If successful in a histologically corroborated trial, immunoscintigraphy would provide a means to focus surgical intervention for lymphadenectomy in the highest-risk patient category. While numerous tumor foci that were missed by standard methods of detection were also demonstrated, detection of these sites was not the objective of our present study, and requires surgical-pathologic confirmation that we

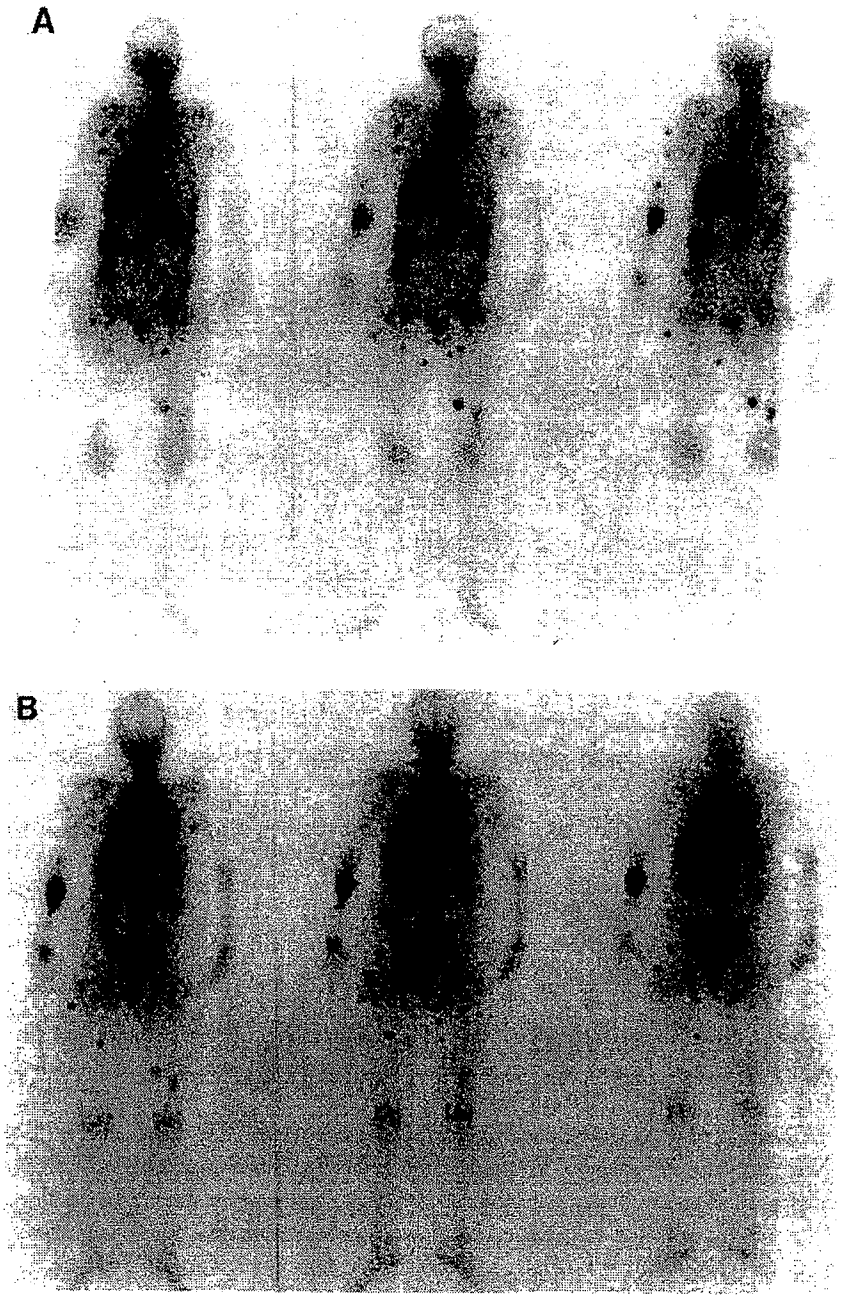

Fig 1. (A and B) See legend on facing page.

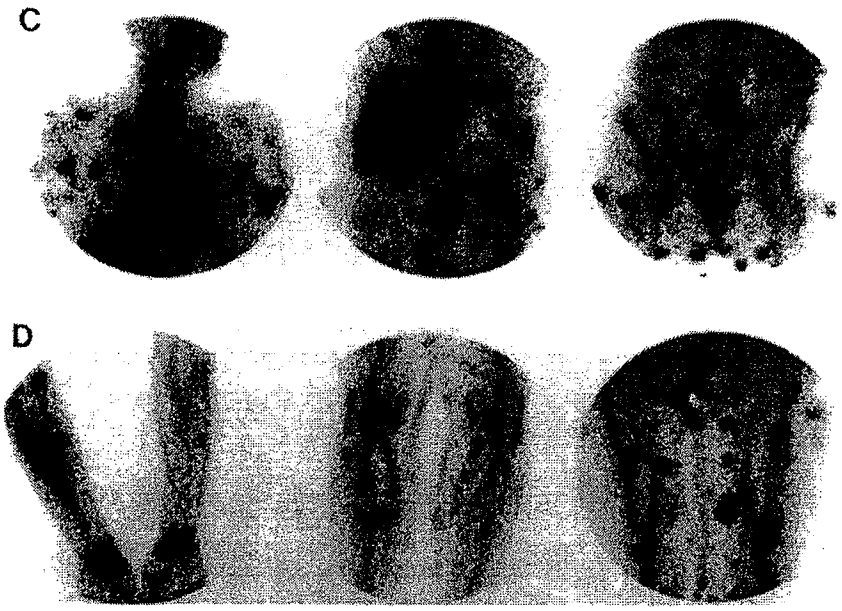

Fig 1. Patient no. 21: tomographic ^{67}Ga images ([A] anterior and [B] posterior), compared with anti-gp240 ^{111}In conjugate images of (C) chest, and upper and lower abdomen, and (D) arms, legs, and thighs. Multiple lesions apparent on antibody image were inapparent on gallium scan, but palpable.

did not undertake during the present antibody study.

A high incidence of axillary uptake in this study may be due to localization in hair bulbs, sweat, or sebaceous glands, as suggested in recent immunochemical studies.[30] In general, the differentiation of false- from true-positives involved a careful correlation of physical examination and imaging studies, especially CT and ^{67}Ga during patient follow-up evaluation. The protocol for scoring of antibody images (as of all tumor imaging studies) required consensus of a team of radiology, nuclear medicine, and medical oncology physicians. This system assured consistency and quality control.

Analysis of the pharmacokinetics of MoAb 96.5 revealed a decreasing volume of distribution at higher doses, and decreased localization in liver.[21] However, it was noted that blood clearance fit an open one-compartment mathematical model, with a T½ in plasma of 31 hours independent of dosage. The study of 96.5 MoAb also revealed no differences between male and female blood clearances, in contrast to our anti-p240 study. Two findings in our study merit discussion: first, the prolonged plasma clearance T½ values observed with increasing total antibody dosages parallel the improved sensitivity for detecting metastatic sites; second, the differences in male and female blood plasma clearance values suggest the presence of a tissue "sink" that is associated with gender, ie, the testes. Imaging studies that showed localization to the testes of male patients suggest to us that testicular tissue binding, rather than hormonal effect of testicular origin, may explain some of the differential phar-

Fig 2. Patient no. 26: (left) gallium and (right) gp240 images of neck and supraorbital soft tissue metastases.

macokinetics observed in this study. Our one study carried out in a castrated male patient with melanoma and a history of prostatic carcinoma, in whom blood clearance T½ values resembling the female pattern were observed, tends to confirm this hypothesis. However, preliminary immunohistological studies (data not shown) suggest only minimal stromal binding of ZME-018 in human testicular tissue.

We conclude that the monoclonal antibody ZME-018, radiolabeled with 5.0 mCi of ^{111}In, is a generally nontoxic immunoscintigraphic reagent that is capable of imaging most metastatic melanoma sites of ≥ 1 cm in size. Comparative analysis of the imaging of melanoma by ^{67}Ga citrate and other antibodies, with detailed biodistribution data, will be published in two subsequent reports.[31,32] Future efforts to improve antibody imaging and therapy of melanoma are in progress, with attention to reduction of background uptake and induction of increased expression of the relevant tumor antigens (using cytokines such as the interferons) in conjunction with antibody administration.

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EXHIBIT 7

[CANCER RESEARCH 45, 4904–4909, October 1985]

Increased Labeling of Human Melanoma Cells *in Vitro* Using Combinations of Monoclonal Antibodies Recognizing Separate Cell Surface Antigenic Determinants

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ABSTRACT

A panel of mouse anti-melanoma monoclonal antibodies (MoAb) were analyzed for reactivity with human melanoma cells singly and in combination. Five MoAb, ZME-018, 96.5, P94, 4.2, and 5.1, reactive with individual cell surface melanoma-associated antigens were tested with seven melanoma cell lines and seven fresh tumor biopsies. Cells were incubated with the MoAb, indirectly stained with fluorescein-conjugated goat anti-mouse immunoglobulin, and analyzed by flow cytometry. Percentage of labeled cells and relative fluorescence intensity (FI) with individual MoAb varied with different cell lines and biopsy samples. The most reactive MoAb, ZME-018, 96.5, and P94, labeled 29–93% of the cells from cell lines with relative FI of 2–59 units, thereby demonstrating phenotypic diversity of these cells. Similar results were obtained with cells derived from tumor biopsies, where 1–73% of cells were labeled and relative FI ranged from 0–27. These variations were reduced by using a "cocktail" of MoAb which recognized different melanoma-associated antigens. In cell lines both the percentage of labeled cells (range, 82–95%) and relative FI (range, 36–115) increased substantially ($P < 0.025$ and $P < 0.005$, respectively) when a "cocktail" prepared from all five MoAb rather than individual MoAb was used. A cocktail of MoAb increased the percentage of labeled tumor biopsy cells (range, 53–78%; $P < 0.01$) and relative FI (range, 11–69; $P < 0.025$). The mean FI obtained by incubating cells with a cocktail of suboptimal concentrations of three MoAb (ZME-018, 96.5, P94) was 48 ± 12 (SD), which was significantly increased compared to the mean FI seen with suboptimal concentrations of MoAb alone (ZME-018, 7 ± 10; 96.5, 8 ± 7; P94, 2 ± 2; $P < 0.005$). These findings were confirmed by radioimmunoassay using a combination of two MoAb, ZME-018 and 96.5. The data suggest that cocktails of MoAb were more effective than single MoAb alone for melanoma tumor cell labeling *in vitro* and might be more effective for tumor imaging and therapy.

INTRODUCTION

The development of hybridoma methodology (1) has greatly increased our ability to detect and characterize tumor-associated antigens. Currently more than 30 MoAb[2] have been developed which recognize individual MAA on human malignant melanomas (2). Several of these MoAb have been evaluated in humans either alone (3, 4) or coupled to radioisotopes for use in diagnostic imaging (5–7).

There are several problems which need to be solved before MoAb can be used effectively for imaging or therapy. One problem is the heterogeneity of surface antigen expression on fresh tumor biopsies (8, 9) and cell lines (10–13). This heterogeneity has also been demonstrated on individual clones of tumor cell lines (10). Variations in antigen expression and density on individual tumor cells within primary and metastatic tumors (14–16) could account for the difficulties in detecting all known lesions *in vivo* using a single radiolabeled MoAb. One approach to this problem might be to use a combination of MoAb which recognize several different or distinct surface antigens and/or antigenic epitopes covering the range of heterogeneous antigen expression.

In this report we compared the binding of a panel of anti-melanoma MoAb reactive with different MAA singly and in combination (cocktail) to cells from melanoma cell lines and fresh tumor biopsies. Our results indicate that both the percentage of tumor cells labeled and the relative amount of MoAb bound per cell were significantly augmented by using MoAb cocktails compared to individual MoAb alone.

MATERIALS AND METHODS

Cell Lines. Melanoma cell lines BMCL, G, M-40, and RON were obtained from Dr. James Bowen, Section of Virology, M. D. Anderson Hospital and Tumor Institute, University of Texas. All of these lines were established in culture from melanoma biopsy specimens; lines G and M-40 produced melanin. Melanoma cell lines C-81-46 and C81-61 were obtained from Dr. Frank Meyskins, University of Arizona Cancer Center. Melanoma cell line 294T is commercially available and was described by Creasey *et al.* (17). Adult human fibroblasts, used as a negative control, were obtained from Dr. Christopher Reading, Department of Tumor Biology, M. D. Anderson Hospital and Tumor Institute. Daudi, a Burkitt's lymphoma cell line established by Klein *et al.* (18), also used as a negative control, was cultured in sterile 500-ml plastic culture flasks (Falcon Plastics, Oxnard, CA) in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 0.5% gentamicin, and 1% (v/v) L-glutamine. Melanoma cells and fibroblasts were initially cultured at a density of 2×10^5 cells/ml in Liebowitz L-15 medium (Gibco) supplemented with 10% fetal calf serum in a humidified incubator at 37°C with 5% CO_2 in air. Cells were routinely split at confluence every 6–7 days and maintained in long term culture. Prior to labeling with MoAb cells were removed from culture flasks by gentle scraping or aspiration (Daudi cells), washed in PBS, and counted. Viability as assessed by trypan blue was greater than 70% in all cell lines tested.

Preparation of Melanoma Tumor Cell Suspension. Fresh unfixed surgical specimens were cut and minced into 1-mm^3 fragments in RPMI media supplemented with 10% fetal calf serum and passed through a

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[2] The abbreviations used are: MoAb, monoclonal antibodies); MAA, melanoma-associated antigens; FI, fluorescence intensity; PBS, phosphate-buffered saline.

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0.38-μm sieve to obtain a single cell suspension. The cell suspension was enriched for viable tumor cells by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation, washed with PBS, and counted. Viability of cells as assessed by trypan blue dye exclusion ranged from 60–95%. Nucleated bone marrow cells obtained from normal donors were purified by Ficoll-Hypaque density gradient centrifugation as above and used as a negative control with the MoAb labeling procedure.

Monoclonal Antibodies. Murine anti-human melanoma MoAb were obtained from Hybritech, Inc., San Diego, CA. All antibodies were produced and purified by Hybritech using conventional hybridoma techniques. MoAb ZME-018 is a murine IgG2a antibody specific for a high molecular weight cell surface MAA (19). A second murine MoAb, subclass IgG2b reacting with a single 94,000 M_r glycoprotein, was also used (20). For purposes of simplicity the MoAb is designated P94 in this study. MoAb 96.5 is murine IgG2a which reacts specifically with P97[a] antigen epitope (21). MoAb 5.1 is specific for MAA P210 and is of the IgG1 subclass (22). MoAb 4.2 is a murine IgM MoAb specific for a ganglioside antigen, expressed on the majority of human melanomas (23). As an irrelevant MoAb control we used a MoAb with a structure and subclass identical to that of MoAb 96.5 but nonreactive with P97 and any other known MAA antigen (obtained from Hybritech, Inc.).

Immunofluorescent Techniques. Fresh tumor cells and cell lines suspended at a concentration of 1–3 × 10^5 cells in 50 μl of PBS were mixed with 50 μl of individual MoAb at concentrations ranging from 1 to 50 μg/ml and incubated for 30 min at 4°C. The final concentration of MoAb ranged from 0.5 to 25 μg/ml. Cells were washed twice with PBS and incubated for an additional 30 min at 4°C with 75 μl of a 1:50 dilution of fluorescein-conjugated F(ab')$_2$ goat anti-mouse immunoglobulin (Kallestadt Laboratories, Austin, TX). The cells were then washed twice with PBS, and the number of cells labeled with MoAb or MoAb combinations as well as the relative fluorescence intensity of labeling (FI) were determined by flow cytometry. The flow cytometer (Spectrum III-Ortho Diagnostic Systems, Westwood, MA) was standardized daily with Fluorotrol-GF (Ortho). Tumor cell populations were determined by narrow forward and right angle light scatter characteristics. Careful gating procedures were used to ensure exclusion of dead cells and lymphocytes (24). Values for FI were controlled by determination of autofluorescence of unstained melanoma cells. Relative FI is reported as units of fluorescence on a scale from 0 to 250.

Radioimmunoassay Procedures. Cells from the melanoma cell line 294T along with Daudi cells were suspended in 15 x 100-mm glass tubes (Fischer Scientific, Dallas, TX) at a concentration of 1–2 × 10^6 cells/tube in 50 μl PBS. One mg each of two MoAb, 96.5 and ZME-018, were labeled with 1 mCi ^{111}In using a modification of the technique of Krezcarek and Tucker (25). A more detailed report of the labeling procedure has been published previously (26). One hundred ng of each MoAb ^{111}In conjugate (*i.e.*, equivalent of 100 nCi ^{111}In or approximately 150,000 cpm) and a cocktail of MoAb in conjugates (*i.e.*, 50 ng each of labeled 96.5 and ZME-018) were incubated with melanoma and Daudi cells in a total volume of 0.1 ml PBS for 30 min at 4°C. Cells were washed twice in PBS and radioactivity was determined using a gamma counter (Packard Instruments, Des Plaines, IL). Blocking studies were also performed by incubating cells with unlabeled MoAb for 30 min, washing twice, and then incubating with the respective ^{111}In labeled MoAb. The actual ng quantities of MoAb bound were calculated using the formula

$$\text{ng MoAb bound} = \frac{\text{cpm bound to cells}}{\text{cpm added}} \times \text{ng MoAb added}$$

RESULTS

Reactivity of Different MoAb Cocktails with Melanoma Cell Lines. In initial experiments optimal binding of MoAb to most cell lines occurred when cells were collected between 6 and 7 days of growth after being subcultured. MoAb reactivity with MAA was less if cells were analyzed prior to 5 days or after 7 days in culture (data not shown). Hence all experiments were performed at a time when maximal MAA expression occurred, to compensate for variations in antigen expression which might be related to cell cycle or culture conditions.

In preliminary experiments we tested the reactivity of two of the five available MoAb (96.5 and ZME-018) against the melanoma cell line 294T at final MoAb concentrations ranging from 0.5 to 25 μg/ml. Reactivity was detected at individual MoAb concentrations ranging from 5 to 25 μg/ml; above 25 μg/ml no further increase in either the percentage of cells labeled with MoAb or the relative FI was observed (data not shown). Hence a 25-μg/ml concentration of each MoAb was used in experiments described below.

Immunoreactivity of five anti-melanoma MoAb with seven different human melanoma cell lines is shown in Table 1. Overall the most reactive individual MoAb were ZME-018, 96.5, and P94. However, there was considerable heterogeneity in MAA expression on the different cell lines with variability in the percentage of labeled cells and FI. For example MoAb ZME-018 showed high binding with all cell lines except RON. Similarly MoAb 96.5 showed high binding with BMCL cells but low reactivity with C81-46 and RON. Of all the MoAb used, only P94 reacted well with the RON cell line. In contrast a cocktail prepared from all five MoAb reacted very well with all cell lines. The percentage of cells labeled by the cocktail were in the range of 82–95% (median, 89%) compared to 29–93% (median, 71%) with ZME-018 alone (25 μg/ml) ($P < 0.025$). The percentage of cells reacting with 96.5 alone (25 μg/ml) ranged from 22–81% (median, 62%), and the range for P94 alone was 31–83% (median, 61%).

The relative FI was also greater with cocktails compared to single MoAb. When cell lines were examined for FI they showed the highest intensity with ZME-018 (range, 9–96 units; median, 49), followed by 96.5 (range, 2–49 units; median, 23) and P94 (range, 4–31 units; median, 18). FI for other MoAb were in the range of 0–38 units. A MoAb cocktail prepared from all five MoAb demonstrated significantly higher FI (range, 36–115; median, 93; $P < 0.005$). A representative example of the percentage of labeled cells and FI using a single MoAb and a cocktail of MoAb is shown in Chart 1.

Improved Labeling of Cell Lines with Suboptimal Concentrations of MoAb in Combination. In the previous experiments all MoAb were used at optimal concentrations (*i.e.*, 50 μl of a 50-μg/ml concentration per 1–3 × 10^5 cells in a total of 0.1 ml of PBS or an equivalent of MoAb of 25 μg/ml. To determine whether increases in the percentage of labeled cells and FI could occur if suboptimal concentrations of MoAb were added, 10 μl of 50-μg/ml dilutions of each of the three most reactive MoAb (ZME-018, 96.5, and P94) were added individually (total, 5 μg/ml) and in combination (total, 15 μg/ml) to the 294T melanoma cell line (Table 2). The percentage of cells labeling with the suboptimal concentration (*i.e.*, 5 μg/ml) of each MoAb was equivalent to those labeling at the 15-μg/ml concentration; however, the relative FI was markedly diminished. The FI increased with a cocktail of three MoAb (total MoAb concentration, 15 μg/ml). The mean FI was significantly greater [48 ± 12 (SD); $P < 0.002$] than the sum of the FI obtained for each MoAb [8 + 7 + 2] added singly

Table 1

Reactivity of MoAb with melanoma cell lines

Melanoma cell lines	Individual MoAb[a]												Cocktail[b] (96.5 + ZME + 94 + 4.2 + 5.1)	
	NR[c]		96.5		ZME-018		P94		4.2		5.1			
	%	FI	%	FI	%	FI	%	FI	%	FI	%	FI	%	FI
RON	0	0	31[d]	2	29	9	81	22	26	20	7	5	89	36[e]
M 40	4	0	72	11	93	49	68	8	11	0	12	2	95	65
294 T	2	0	62	31	62	59	61	18	27	33	22	4	86	93
G	3	1	67	24	79	52	31	19	60	8	40	9	89	112
BMCL	5	0	81	49	71	39	83	16	48	20	11	0	92	97
C 81-46	11	15	22	23	76	96	36	31	39	38	28	24	82	115
C 81-51	6	1	59	4	59	28	43	4	29	5	7	1	88	41
Control cell lines														
Fibroblasts	0		1		5		1		0		5		5	
Daudi cells	0		1		0		1		1		0		0	
Bone marrow	2		2		1		0		0		0		0	

[a] Concentration of MoAb, 25 μg/ml/1–3 × 10^6 cells.
[b] Mixture of five MoAb at a total concentration of 25 μg/ml/1–3 × 10^6 cells.
[c] NR, nonimmunoreactive MoAb. This MoAb was similar to 96.5 in subclass IgG2a.
[d] Represents mean of duplicate or, in the case of M 40 cell line, triplicate experiments.
[e] Differences between single MoAb and cocktail are statistically significant for labeling ($P < 0.025$) and FI ($P < 0.005$). Paired *t* test was used for statistical evaluation.

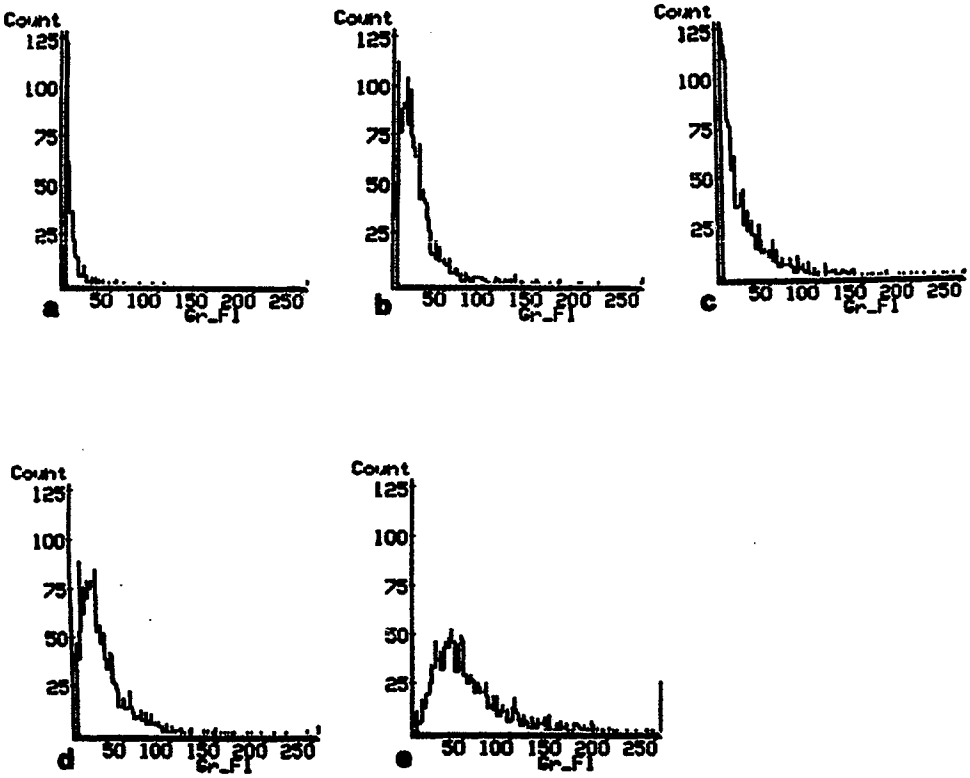

Chart 1. *a*, flow cytometry of M-40 cell line reacted with FITC-GAMig alone (control). *Ordinate*, cell number; *abscissa*, green fluorescence intensity (*Gr-FI*); *b*, same histogram showing reactivity of ZME-018 MoAb; *c*, histogram demonstrating reactivity of 96.5 MoAb; *d*, reactivity with MoAb cocktail (P94, ZME-018, 96.5). Percentage of cells labeled and FI improved over ZME-018 or 96.5; *e*, reactivity with cocktail of 5 MoAb (P94, ZME-018, 96.5, 4.2, 5.1).

at individual concentrations of 15 μg/ml. Higher individual concentrations of MoAb in the cocktail (*i.e.*, 15 μg each) did not improve either the mean percentage of cells labeled (82 ± 6) or the mean FI (49 ± 6) over a MoAb cocktail made up of 5-μg/ml concentrations of each MoAb.

Similar results were obtained using ng amounts of ^{111}In-labeled MoAb in a radioimmunoassay (see "Materials and Methods"). As shown in Table 3 500-ng/ml quantities of a combination of two MoAb (ZME-018 and 96.5) bound to 294T to a greater extent (12.97 ng) than did 1000-ng/ml amounts of each MoAb alone (4.75 and 5.16 ng, respectively; $P < 0.0001$). Binding to cells was effectively blocked if cold antibody was first added followed by the respective labeled antibody. Of further interest was the finding that addition of cold unlabled 96.5 followed by ^{111}In-labeled ZME-018 increased binding of ZME-018 (11.75 ± 5.54 ng) over that which occurred for ^{111}In-labeled ZME-018 alone (4.75 ± 1.70 ng).

Reactivity of Different MoAb and MoAb Cocktails with Single Cell Suspensions of Melanoma Tumors Derived from Human Biopsy Materials. The pattern of reactivity of different

Table 2

Reactivity of T294 with individual anti-melanoma MoAb and cocktail of MoAb

MoAb concentration (μg/ml)	96.5		ZME-018		P94		Cocktail	
	%	FI	%	FI	%	FI	%	FI
5	59 ± 3[a]	8 ± 7	50 ± 7	7 ± 10	47 ± 10	2 ± 2	81 ± 4	48[b] ± 12[c]
15	63 ± 9	12 ± 2	51 ± 5	11 ± 13	48 ± 4	7 ± 10	82 ± 6	49[d] ± 6

[a] Mean ± SD of five experiments.
[b] Addition of 5-μg/ml concentrations of each MoAb for a total of 15 μg/ml.
[c] Differences between single MoAb and cocktail are statistically significant for percentage of cells labeling ($P \leq 0.005$) and FI ($P < 0.002$). Student's test was used to compare mean values.
[d] Addition of 15-μg/ml concentrations of each MoAb for a total concentration of 45 μg/ml.

Table 3

Quantitative binding of MoAb to T294 melanoma cell line

MoAb combination	Total concentration[a] of MoAb added (ng/ml)	cpm	Mean ± SD MoAb amount bound (ng)
^{111}In-96.5	1,000	10,322 ± 1,145[b]	5.16 ± 0.57
^{111}In-ZME-018	1,000	9,508 ± 3,422	4.75 ± 1.70
^{111}In-96 + ^{111}In-ZME-018	1,000	25,955 ± 10,618	12.97 ± 5.30[c]
96.5 + ^{111}In-96.5	2,000	1,087 ± 1,321	0.54 ± 0.66
ZME-018 + ^{111}In-ZME-018	2,000	670 ± 723	0.33 ± 0.36
ZME-018 + ^{111}In-96.5	2,000	12,290 ± 2,760	6.13 ± 1.40
96.5 + ^{111}In-ZME-018	2,000	23,516 ± 11,084	11.75 ± 5.54

[a] We incubated 150,000 cells in triplicate with appropriate MoAb (1000 ng/ml) and ^{111}In (1000 nCi/ml). A representative example of three identical experiments is shown.
[b] Mean ± SD of triplicate samples.
[c] Significant augmentation of binding compared with each MoAb alone ($P < 0.00001$); compared with the sum of each MoAb ($P < 0.025$).

fresh tumor biopsies and MoAb is demonstrated in Table 4. Mixtures of two, three, or five MoAb were compared to each MoAb added alone. A total of seven individual tumor cell suspensions were evaluated. Several distinct patterns of reactivity emerged. In sample 1 the majority of labeled cells expressed the antigens P97 and high molecular weight MAA and hence demonstrated good labeling with either 96.5 or ZME-018. Therefore a mixture of other antibodies along with 96.5 and ZME-018 increased only the relative FI and did not increase the percentage of labeled cells. In sample 2 cells reacted predominantly with MoAb 4.2. Similar to the above example the addition of the four other MoAb in a cocktail increased only the relative FI and not the percentage of labeled cells. A heterogeneous population of tumor cells was found in samples 3, 4, and 7, and low expression of all five MAA as recognized by MoAb was present. In contrast to the previous samples a mixture of MoAb recognizing all five MAA increased both the percentage of labeled cells and the relative FI. In sample 6 there were two populations of cells, one predominantly labeling with P94 and the other with ZME-018. A combination of these MoAb increased the percentage of labeled cells to the same level as a combination of all five MoAb. In all samples tested the binding of the MoAb cocktails was higher than that of single MoAb alone. A cocktail prepared from two MoAb, ZME-018 and 96.5, showed less reactivity than a cocktail prepared from three MoAb. In general MoAb cocktails prepared from five MoAb showed higher reactivity than cocktails prepared from three MoAb.

DISCUSSION

A variety of MoAb reactive with MAA have been described (2, 9, 19–23). In this report we analyzed the ability of a panel of MoAb to react singly and in combination with a panel of melanoma cell lines and fresh tumor samples. Most of the melanoma cell lines used had a high expression (*i.e.*, from 60–80%) of three previously defined antigens, high molecular weight MAA, P97, and P94. Other antigens, 5.1 and 4.2, were expressed to a lesser extent. Considerable heterogeneity in MAA expression existed among the cell lines and fresh tumor samples.

Phenotypic diversity of cancer cells is a well described phenomenon (15, 16). Burchiel *et al.* (11, 12) analyzed binding of MoAb recognizing HLA-AB antigens, Ia-like antigens, and two MAA (M_r 280,000 and 94,000) in human melanoma cell lines by flow cytometry. Considerable heterogeneity with regard to MAA antigen expression was found which correlated to a limited extent with cell size and cell cycle. Both the M_r 280,000 and 94,000 MAA were differentially expressed during the cell cycle with maximal expression occurring during G_2 + M phases. In another study (10) melanoma cell lines were cloned in methylcellulose and expanded in liquid culture; the antigenic profile of these metastatic tumor cell clones was analyzed using a panel of eight MoAb to MAA. Both a quantitative and a qualitative heterogeneity of antigen expression was observed among and within the different clones in spite of the cloning procedure. Heterogeneity of antigen expression was also described by Houghton *et al.* (27) using bulk cultures of melanoma cells. The authors attributed changes in antigen expression as arising along a melanocyte differentiation pathway. Similar results have been published for other tumor cell lines, particularly oat cell and squamous cell lung cancer (28).

The most important finding of this study was that variations in antigen expression for a single MoAb could be significantly reduced by using a "cocktail" of MoAb which recognized a series of different MAA. Both the percentage of cells which bound MoAb and the actual quantity of MoAb bound, as assessed by relative FI on flow cytometer, increased significantly in both cell lines and fresh tumor cells. In this instance the relative FI histogram is a rough measure of antigen density; however, it cannot discriminate individual "brightly staining" cells from an overall increase in antigen density per cell. A second important observation was that the percentage of labeled cells and more significantly the relative FI was increased substantially if suboptimal concentrations of three MoAb with relatively high affinity (ZME-018, 96.5, P94) were added to cells rather than saturating concentrations of each MoAb alone or combined. In this case the mean FI with suboptimal amounts of MoAb in the cocktail was actually higher than the sum total FI expected for each MoAb added individually in optimal concentrations suggesting a synergistic effect (Table 2).

This represents one of the first studies to examine the efficacy

Table 4

Reactivity of MoAb against malignant melanoma cells derived from human biopsy materials

	Individual MoAb										Cocktail[a]					
	96.5		ZME-018		P94		4.2		5.1		96.5 + ZME		96.5 + ZME + P94		96.5 + ZME + P94 + 4.2 + 5.1	
Biopsy	%	FI	%	FI	%	FI	%	FI	%	FI	%	FI	%	FI	%	FI
1	73[b]	22	73	17	61	2	45	12	17	8	ND[c]	ND	78	30	78	38
2	37	0	53	23	35	0	70	37	22	0	67	36	ND	ND	67	69
3	58	19	27	8	51	8	38	25	21	7	61	21	65	27	70	30
4	6	12	6	17	11	19	40	23	6	16	ND	ND	ND	ND	55	45
5	18	10	28	23	42	27	ND	ND	ND	ND	51	37	ND	ND	53	43
6	1	5	53	5	47	0	ND	ND	ND	ND	ND	ND	77	14	78	23
7	23	8	22	14	41	9	ND	ND	ND	ND	33	14	44	20	66	11

[a] Individual antibody concentrations were 25 μg/ml as were total concentrations of antibodies in cocktails.
[b] Represents an individual experiment.
[c] ND, not done.

of using cocktails of MoAb reacting with distinct MAA to improve labeling of melanoma cell lines and fresh tumor cells *in vitro*. Dantas *et al.* (29) were able to detect melanoma cells in bone marrow using a combination of three MoAb which recognized separate epitopes on P97: 96.5, 8.2, and 118.1. Preliminary work by Colnaghi *et al.*[3] demonstrated that a panel of MoAb reactive against breast (30) and ovarian carcinoma (31) significantly augmented the sensitivity of detecting both circulating antigens and metastatic tumor cells in bone marrow over individual MoAb used alone. Ceriani *et al.*[4] recently demonstrated that a cocktail of anti-breast cancer MoAb appeared to be more effective in eradicating tumor cells in nude mice than was single MoAb alone.

Binding of ng quantities of a combination of two MoAb, ZME-018 and 96.5, to T294 cells was significantly greater ($P < 0.00001$) than each MoAb alone as well as the sum of both antibodies ($P < 0.025$) as determined by a sensitive radioimmunoassay technique (Table 3). Of additional interest was the observation that quantitative binding of ^{111}In-labeled ZME-018 MoAb was increased substantially if unlabeled 96.5 was first added followed by ^{111}In-labeled ZME-018. Although the explanation for this phenomenon is unclear it suggests that one MoAb, depending on its structure or other characteristics, may actually augment the binding of a second MoAb, perhaps by exposing cryptic antigen sites through cross-linking, etc. Further studies are needed in an attempt to understand the mechanisms involved.

Similar findings were also observed using fresh tumor cell suspensions (Table 4). In contrast to data using cell lines P94 bound much more avidly to fresh tumor cells. The reason for this difference is uncertain although it is possible that more fresh tumor cells were in G_1-G_2 phase transition than cell lines (11). In general the degree of binding of MoAb cocktails to fresh tumor suspensions was reflected by the affinity of each MoAb for its respective MAA. For example if both ZME-018 and 96.5 bound individually to over 80% of cells, the percentage of cells labeled did not increase substantially with a cocktail of both MoAb. On the other hand if MoAb bound to a lesser extent individually, the percentage of cells and more importantly the FI increased significantly if a cocktail of the respective antibodies was used.

In conclusion the findings reported above could have considerable importance in the clinical setting. In view of the heterogeneity within tumors it is likely that a cocktail of MoAb could be more efficacious for tumor imaging and/or therapy. However, one should be cautious in overinterpreting our results to imply that "more is better." It is likely that either optimal or suboptimal combinations of MoAb exist depending on their respective affinities and interactions when used in combination; hence certain MoAb might either inhibit or augment binding of another MoAb depending on their individual characteristics. Also pharmacokinetic considerations and nonspecific uptake may influence the binding of single MoAb *versus* cocktails *in vivo*.

[3] M. I. Colnaghi, S. Canevari, S. Bernard, S. Miotti, and F. Rilke, unpublished data.
[4] R. L. Ceriani, unpublished data.

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RELATED PROCEEDINGS APPENDIX

There are no related proceedings.